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RecBCD-Mediated Replication and Recombination in *Escherichia coli*

by

MOHAMMAD REZA MOTAMEDI ©

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fulfillment of the requirements for the degree of Doctor of Philosophy

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Mohammad Motamedi

Permanent Address:

#408, 945 Marine Drive
West Vancouver, B.C.
V7T 1A8

Dated: Aug 30 / **2000**

Abstract

DNA double-strand break-repair (DSBR) is accomplished by homologous recombination in many organisms. In *Escherichia coli,* except under special circumstances, DSBR was thought to result from breakage and reunion of parental DNA molecules, assisted by known Holliday junction processing proteins RuvABC and RecG. In Chapter 2 of this thesis, I provide physical evidence for a major alternative mechanism in which replication copies information from one chromosome to another generating recombinant molecules *in vivo.* This alternative mechanism can occur independently of Holliday junction processing proteins, requires DNA polymerase III, and produces recombined DNA molecules that carry newly replicated DNA. The replicational mechanism underlies about half the recombination of linear DNA in *E. coli*; the other half occurs by breakage and reunion, which is shown to require resolvases, and is replication-independent.

In Chapter 3 of this thesis, I dissect the role(s) of each of the HJ processing proteins in the context of the two DSBR pathways in *E. coli.* I find physical evidence that the RuvA, RuvB, and RuvC proteins all are required for break-join DSBR. In *recG* cells, break-join recombination is reduced significantly, indicating that the RuvABC-dependent break-join mechanism requires RecG for its optimal efficiency. This provides the first direct physical evidence that RuvABC and RecG work together, catalyzing break-join recombination reactions *in vivo.* Interestingly, even though there is a significant decrease in break-join recombination in *recG* cells, the overall recombination frequency remains unaffected. This results from a RuvABC-dependent replicative mechanism that restores the recombination frequency to wild-type levels in these cells. In support of this, I find that the absence of any HJ processing proteins, leading to the accumulation of unprocessed recombination intermediates, promotes

replication dramatically. Based on these results, a model is proposed in which two distinct DNA intermediates occur during DSBR *in vivo*. One is processed exclusively by RuvABC, sometimes acting in concert with RecG, whereas the other is processed *via* DNA synthesis, independently of any HJ processing proteins.

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled RecBCD-mediated Recombination and Replication in *Escherichia coli* submitted by Mohammad Reza Motamedi in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Molecular Biology and Genetics.

Dr. Ross Hodgetts

Claire Cupples

Dr. Claire Cupples

Dr. Susan Lees-Miller

D Murray

Dr. David Murray

Linda Reha-Krantz

Dr. Linda Reha-Krantz

Michael Weinfeld

Dr. Michael Weinfeld

Dated: Aug 2 2000

In the memory of the late Mohammad Reza Pahlavi, the Shah of Iran, whose vision of a strong modern Iran shall forever live in my heart. His selfless quest for a prosperous Iran was achieved through a marriage between new technology and ancient history. He is the source of my inspiration. It is because of his efforts that my life, my accomplishments, including this thesis, are possible.

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Chapter 1

General introduction

Homologous recombination is a process in which two similar DNA molecules exchange pieces. Only DNA molecules that share greater than 80 percent sequence identity (the criteria for considering molecules homologous with each other) can undergo homologous recombination. Non-homologous DNA molecules are prevented from general recombination by mechanisms that are not yet fully understood. The exchange can take place anywhere along the length of the two molecules and is thus considered general. This process is precise, such that normally no associated deletion or addition of nucleotides is detected at the borders of the exchange, preventing the loss of any genetic information (For general reviews,22rg and Motamedi 1999).

Roles of recombination

Recombination is essential for many biological functions in cells and is hence conserved in evolution: many of the recombination enzymes identified in prokaryotes have direct homologues in higher eukaryotes, including humans (Kanaar and Hoeijmakers 1998; Thacker 1999a; Thacker 1999b). One of the important functions of recombination is to repair DNA double-strand breaks (DSBs) in cells. DSBs are common DNA lesions that arise by a variety of routes in all living cells (e.g. Skalka 1974; Michel et al. 1997; Michel 2000). These lesions are also the starting (or an intermediate) substrate for many of the homologous recombination reactions that occur in cells (Stahl 1986; Keeney et al. 1997; Paques and Haber 1999; Rosenberg and Motamedi 1999; Kowalczykowski 2000). Because DSBs interfere with important cellular functions (such as replication), different mechanisms have evolved for their repair. The most accurate method for repairing DSBs is by homologous recombination. Recombination is used to swap DNA pieces precisely, such that DSBs are repaired without the loss of any genetic information or accompanying chromosomal translocations. This contrasts with another method for repairing DSBs, simple ligation of available DNA ends, also known as non-homologous end-joining, which often results in significant loss of genetic material and gross chromosomal changes (Tsukamoto and Ikeda 1998; Haber 1999). The repair of DSBs via recombination, also known as double-strand break-repair (DSBR), is used by many organisms and is required for normal functioning of cells (Kuzminov 1995; Kanaar and Hoeijmakers 1998; Paques and Haber 1999; Kowalczykowski 2000).

Recombination also operates to preserve genomic stability (e.g. Ellis et al. 1995), create new linked combinations of alleles, restart collapsed replication forks (reviewed in Michel 2000), promote some mutations in bacteria and yeast (reviewed in Rosenberg et al. 1998), carry out mating-type switching in yeast (Haber 1998), and ensure proper segregation of chromosomes during meiosis (e.g. in Drosophila Bopp et al. 1999; fission yeast Krawchuk et al. 1999; and C. elegans Zetka et al. 1999). Thus, its functioning is essential for many biological activities. Excessive recombination threatens the integrity of the genome, and has been shown to correlate with premature aging and cancer (e.g. Ellis et al. 1995; Yu et al. 1996). The absence of recombination decreases cell viability and resistance to DNA damaging agents (such as UV light) and prevents synapse of homologous chromosomes in meiosis thus impeding their proper segregation. Thus a balance between factors that promote and inhibit recombination is critical for its proper biological functioning in cells. Moreover, recombination fuels evolution by creating new combinations of linked alleles in the offspring. This is a major source of genetic variability and gives organisms the potential to respond and adapt to their changing environment.

Early models for recombination

The quest to understand the molecular mechanisms of homologous recombination has a long history in science. In fact, recombination is one of the oldest subjects in genetics, its discovery dating back to 1909 (reviewed in Kuzminov and Stahl 1999): it was initially discovered cytologically, as the exchange of chromosome arms during meiosis. The first general model for homologous recombination emerged following this observation, and later became known as the break-join model for genetic exchange. Since that time, two other general models for recombination have been proposed (see Figure 1-1 and reviewed below).

According to the break-join recombination model, exchange between two homologous chromosomes occurs when the chromosomes are cut at the same site, and the resulting pieces are shuffled and ligated, forming a hybrid molecule carrying genetic information from both parental chromosomes (see Figure 1-1A). A testable prediction of this model is that the product of this type of recombination should contain genetic material only from the parental chromosomes, with no requirement for new DNA synthesis. This is in contrast to the type of recombination products predicted to form by the other two models for recombination (see below), which invoke chromosome duplication as a necessary step in genetic exchange.

The second general model for homologous recombination, copy-choice, was proposed by Belling in the 1930's (referenced in Kuzminov and Stahl 1999). According to this model, DNA replication is used to recombine DNA: recombination occurs when the replication forks of two homologous chromosomes switch templates, copying information from the other molecule (Figure 1-1C). This model predicts that recombinant chromosomes are a hybrid of the two parental molecules, but are made entirely from newly replicated DNA material. Even though this model is no longer pursued, it made a critical contribution to the study of recombination (and DNA replication) by connecting the processes of replication and recombination for the first time.

The break-copy model for homologous recombination emerged as a hybrid of the two aforementioned schemes: a break in a chromosome initiates DNA replication, copying information from the homologous partner (Figure 1-1B). This implies that recombination intermediates may serve as initiating signals for DNA replication and that by replicating to the end of the chromosome the recombination event is completed. (i. e. replication completes the recombination event.) This way, the recombinant molecule is a hybrid of old and new chromosomal material linked together at the site where chromosome replication is initiated (Figure 1-1B).

Overall, the early models of recombination differ in the use and extent of DNA replication in forming the recombinant molecule: the copy-choice model proposes that chromosome replication recombines DNA; the break-copy model assumes that recombination intermediates initiate replication; and in the break-join model, chromosome replication is completely ignored and recombination is thought to occur by the cutting and ligation of chromosome pieces exclusively. Because the three models for recombination make specific predictions about the type of recombination products formed by each hypothesized mechanism, the examination of recombination products for new DNA synthesis could be used to distinguish among the three models. Furthermore, these pathways must be genetically distinguishable; for example, enzymes that are used for DNA replication are likely to be required for break-copy recombination, whereas they are predicted to play no role in break-join recombination. In chapter 2 of this thesis, I tested these models for recombination in the bacterium *E. coli* using techniques to isolate recombinant DNA from cells and examining them for newly replicated and old, parental DNA.

Test of the recombination models

Meselson and Weigle tested the three models for general recombination (Meselson and Weigle 1961; reviewed in Stahl 1986) in *E. coli* using bacteriophage lambda (λ) as the DNA substrate for recombination. In order to determine which mechanism(s) operate in recombination, they developed an assay in which the interval of recombination and extent of new DNA synthesis could be examined and studied simultaneously on the same DNA molecule. One of the advantages of λ is that it has a small, tractable genome, with many well-defined alleles of genes along the length of its chromosome that could serve as genetic markers for determining the interval of recombination. Recombinants can be scored easily, in different intervals, along the λ chromosome, while monitoring new DNA synthesis associated with their recombination.

The detection of new DNA synthesis is possible because all λ virions have a unique density. λ packaging is precise (Kobayashi et al. 1982; Kobayashi et al. 1983) such that exactly a complete λ genome (from *cos*, the packaging origin, to *cos*) is packaged into a unit-size protein coat. (This is in contrast to, for example, phage T4 in which the ends of the chromosome are redundant because packaging incorporates chromosomes that are larger than the length of the T4 genome by 3%) . This feature of λ was used by Meselson and Weigle to develop an assay in which old and newly replicated DNA could be separated physically from each other. This was accomplished by using heavy isotopes of carbon (^{13}C) and nitrogen (^{15}N) to grow λ stocks such that the DNA and the protein coat of these phage would be made from the heavy isotopes. λ crosses were then performed in *E. coli* cells grown in the presence of light isotopes, such that any new DNA or protein synthesis would incorporate light isotopes. Because λ has a defined density, incorporation of light nucleotides as a result of DNA synthesis prior to packaging can be detected in a density gradient of the progeny phage. Moreover, parental (heavy) phage can be distinguished from progeny (light) phage based on their density: the parental phage have heavy protein coats and heavy DNA, whereas all progeny phage have light protein coats and, based on the extent of replication experienced by DNA prior to packaging, light or heavy DNA.

In these experiments, Meselson and Weigle were fortunate to find a small subclass of progeny phage whose chromosomes had not experienced any replication during the lytic cycle. The unreplicated λ progeny contained new (light) protein coats (because the cross was done in the presence of light isotopes), but heavy (HH) chromosomes, and were thus separable from replicated λ progeny with heavy-light (HL) and light-light (LL) DNA. Meselson and Weigle examined the unreplicated progeny subclass for recombination and found recombinant molecules that were made of only parental DNA, indicating that break-join recombination occurs in *E. coli* when λ is used as the DNA substrate. Later, with the discovery of λ recombination systems (Echols et al. 1968; Singer and Weil 1968; Weil and Singer 1968), it became apparent that no final conclusions could be made about the *E. coli* recombination system from these experiments, because λ recombination pathways were also operating in these crosses. But the techniques developed by Meselson and Weigle proved to be critical for my analysis of the involvement of replication in DNA recombination in *E. coli* (Chapter 2).

Further refinement to this technology in the form of controlling the amount of replication experienced by λ, and the use of special λ strains that were defective for their own recombination systems (Russo et al. 1970; Stahl and Stahl

1971a; Stahl and Stahl 1971b), led to the discovery of experimental conditions in which unreplicated phage progeny were the significant, or even the only, progeny recovered from cells, and the *E. coli* recombination system the only pathway for exchanging pieces of λ chromosome (McMilin and Russo 1972). Under these conditions, λ was used to examine *E. coli* recombination in the complete absence of DNA replication. Collectively, these experiments ruled out replicative models as the exclusive route to recombination and provided physical evidence for the occurrence of break-join recombination in *E. coli*. Even though break-copy was not ruled out as a mechanism for recombination (Siegel 1974), break-join was considered to be the major route for recombination in *E. coli* (e.g. Thaler and Stahl 1988; West 1992; Kowalczykowski et al. 1994). This view was further supported by the discoveries of endonucleases, whose function is to specifically cleave recombination intermediates and to complete a break-join recombination event. But a plethora of recent evidence points to the involvement of DNA replication in recombination. This will be discussed in detail below and in Chapter 2 of the thesis. (Also, see the April 2000 issue of *Trends in Biochemical Sciences* for a comprehensive review of this subject as the entire issue is dedicated to the DNA replication and recombination interface.)

The RecBCD System of Recombination in *E. coli*

The molecular mechanism of homologous recombination has been studied extensively in *E. coli*. Over 20 enzymes have been identified to be involved in this process (reviewed in West 1992; Clark and Sandler 1994; Kowalczykowski et al. 1994; Kowalczykowski 2000); however, only a few appear to play key roles in the main pathway of recombination and DSBR, the RecBCD system (reviewed in Eggleston and West 1996). A model for RecBCD-mediated recombination is shown in Figure 1-2. Even though a variety of recombination models exist for this pathway, all share the following features:

1. RecBCD, a heteromultimer of RecB, RecC and RecD proteins, functions as a double-strand DNA (dsDNA) exonuclease, DNA helicase, and ATPase (reviewed in Kowalczykowski et al. 1994). The substrate for RecBCD is a dsDNA end (Figure 1-2A). RecBCD binds to a dsDNA end and travels along DNA unidirectionally, promoting recombination at a low uniform rate until it encounters a special octameric DNA sequence called Chi (Figure 1-2B), cross-over hot-spot instigator (5'GCTGGTGG3') (reviewed in Myers and Stahl 1994; Rosenberg and Motamedi 1999).

2. RecBCD can recognize Chi, only if it encounters it from the GG 3′ end. RecBCD then promotes recombination at Chi and downstream of Chi by its helicase/exonuclease activity, generating single-strand (ss) DNA molecules which are the substrate for RecA protein (Figure 1-2C).

3. RecA catalyzes strand-exchange reactions in *E. coli* (reviewed in Roca and Cox 1997) as do its homologues in eukaryotes (Aravind et al. 1999; Thacker 1999b). ssDNA coated with RecA invades a homologous duplex (Figure 1-2D), forming a heteroduplex recombination intermediate (e.g. a Holliday junction, HJ).

4. These intermediates are substrates for a set of enzymes whose jobs are to process the intermediate into a mature recombinant DNA (Figure 1-2E and F), completing the recombination reaction (reviewed in West 1994).

Holliday junction (HJ) processing in the RecBCD system

In *E. coli*, the RecBCD system uses two apparently independent pathways to process recombination intermediates: the RuvABC and the RecG (Lloyd 1991; reviewed in West 1994). The RuvABC system is composed of RuvA, RuvB and

RuvC proteins. Genetical and biochemical studies suggest that the three proteins work together, physically interact with each other, and form a complex (RuvABC) in vivo and in vitro. *ruvA*, *ruvB*, and *ruvC* mutants display the same phenotypes: a moderate decrease in recombination proficiency (as measured by conjugational and transductional recombination assays) and resistance to DNA damaging agents such as UV light (see Chapter 3 and Lloyd et al. 1984; Sharples et al. 1990). The Ruv phenotype can be rescued by the expression of one protein, RusA (a HJ resolvase), suggesting that *ruv* defective strains are deficient for the same enzymatic function (Mandal et al. 1993). In vitro experiments also have demonstrated direct physical interaction and catalytic synergy between the different components of the proposed RuvABC complex (Hiom and West 1995; Whitby et al. 1996; Eggleston et al. 1997; van Gool et al. 1998; van Gool et al. 1999). These data strongly argue that the three proteins act together as a complex in vivo.

The catalytic activity of each subunit has been studied extensively. RuvA binds HJs and recruits RuvB forming a RuvAB-HJ complex in vitro (Hiom and West 1995). RuvB is an ATP-dependent hexameric DNA helicase, shown to branch-migrate HJs (via its helicase activity) in the presence of RuvA (Hiom and West 1995). The branch migration activity of RuvAB proteins may operate to stabilize and extend heteroduplex DNA, as depicted in Figure 1-2 E. RuvC is a HJ-specific endonuclease that specifically cleaves the DNA strands of a four-way junction (Figure 1-2F), required for the resolution of HJs (Dunderdale et al. 1991; Iwasaki et al. 1991; Bennett et al. 1993). This activity is thought to be critical for the processing of HJs, and is thus implicated strongly in the break-join pathway of RecBCD-mediated DSBR in *E. coli* (for a view of their role in DSBR, see West 1992; West 1994; Kuzminov 1996).

RecG is an ATP-dependent junction specific helicase, thought to operate independently of the RuvABC proteins. This protein is implicated in HJ processing via genetic and biochemical studies. Cells defective for either Ruv or RecG proteins exhibit a mild decrease in recombination proficiency and UV resistance, where as *ruv recG* strains display severe recombination deficiency and UV sensitivity (for a review, see Kuzminov 1996), characteristic of cells defective for homologous recombination (e.g. *recA* strains) (Lloyd 1991). Moreover, RecG is functionally analogous to RuvAB (Lloyd and Sharples 1993b; Lloyd and Sharples 1993a) and is thought to process recombination intermediates, independently by reverse branch migration of HJs (Whitby et al. 1993), or perhaps with the help of another (yet unidentified) HJ-specific endonuclease (Mandal et al. 1993).

The evidence presented in Chapter 2 challenges the exclusivity of a break-join mechanism for DSBR in *E. coli* and indicates a new pathway for recombining DNA, using DNA replication (e.g. Figure 1-1B). In Chapter 3, I re-evaluate the role of each HJ processing protein in DSBR in the context of the two RecBCD-mediated recombination pathways in *E. coli* and discover that both Ruv and RecG proteins are required for the efficient operation of the break-join mechanism. RecG is required for the optimal efficiency of RuvABC; in its absence fewer break-join recombinants are observed. This is the first evidence demonstrating an interaction between the two mechanisms previously thought to act independently in HJ processing.

Future analysis of the results (discussed in Chapter 3) revealed a new model for recombination, in which two distinct recombination intermediates occur in vivo: one is resolved exclusively via the RuvABC acting in concert with RecG, while the other is processed via DNA replication and independently of Ruv or RecG proteins.

Lambda makes a protein which is a HJ resolvase

Interestingly, λ also encodes a HJ processing protein. This was discovered serendipitously by Lloyd's group when searching for suppressers of *ruvA, ruvB,* or *ruvC* mutations. They discovered a gene encoded in an *E. coli* cryptic lambdoid prophage, *qsr,* which is not normally expressed in *E. coli* (Mandal et al. 1993). Most *E. coli* strains (e.g. K-12) harbor a number of different defective prophages, which are thought to have been acquired following lysogeny and subsequent mutations to essential genes of the prophage required for its lytic growth. Defective prophages often are not expressed and perform no function in wild-type *E. coli,* but sometimes can become activated following infection by a lambdoid phage (reviewed in Campbell 1996). The expression of this gene, *rusA,* caused by a promoter-on mutation upstream of the reading frame, suppressed the *ruv* mutation (Mandal et al. 1993; Mahdi et al. 1996). The subsequent cloning of the *rusA* and the neighboring open reading frames revealed a remarkable conservation of genomic organization between this region of the *E. coli* genome and a specific region of the lambdoid family of phages. In λ, the corresponding region is known as *nin,* spanning nine open reading frames including *ninG* or *rap,* which directly corresponds to the *rusA* open reading frame in the *qsr* region of *E. coli* (Mahdi et al. 1996). The *rap* gene had been previously shown to function in recombination; however nothing was known about its catalytic activity at that time (Hollifield et al. 1987; Stahl et al. 1995). The purified Rap protein of λ was later shown to behave as a junction-specific endonuclease, analogous to the RuvC protein of *E. coli* (Sharples et al. 1998). In Chapter 2, we show that in the presence of *nin,* the overall recombination frequency remains the same in *ruv recG* cells compared to *rec*$^+$, indicating that a *nin*-encoded function (perhaps Rap) substitutes for Ruv and RecG deficiencies.

Therefore, I examined the role of Ruv and RecG proteins in DSBR independently of λ-encoded HJ resolution activity by using phages that carry a deletion in the *nin* region. This way HJ resolution can occur only via the *E. coli* Ruv or RecG systems. As a further note, in order to study *E. coli* RecBCD-mediated recombination, all the phages used in this thesis were defective for λ recombination systems and the Gam protein, the specific inhibitor of RecBCD. This way, recombination of λ DNA can be used to assay the host RecBCD system.

T4 recombination and replication

The interconnection between DNA replication and recombination is best characterized in bacteriophage T4. Early pioneering work demonstrated the direct interplay between DNA recombination and replication in the life cycle of the virus: normal DNA replication requires homologous recombination functions (reviewed in Kreuzer 2000). This was shown genetically as mutations in phage-encoded recombination genes caused a "DNA-arrest" phenotype, a defect in which replication initiates normally, but then immediately stops (see Mosig 1998). This and other results were explained by a replicative recombination model in which 3'-ended strand invasions into a homologous duplex form a D-loop (e.g. Figure 1-2E), which was proposed to initiate phage DNA replication (Mosig et al. 1984). These data provided many important clues about the molecular mechanism of replicative recombination. For example, chromosome ends were shown to be the preferred site for most recombination events and require extensive DNA replication for the formation of recombinant chromosomes (Mosig et al. 1984; Mosig 1998). However, because T4 uses its own replication and recombination proteins exclusively (not those of *E. coli)* and the T4 recombination model contradicted the prevailing break-join models at the time, it was suspected that this process was specific to T4 and was dismissed as a general model for many years. Recently, with the accumulation of indirect evidence for the existence of replicative recombination mechanisms in other model organisms (including *E. coli*), the T4-based models are been re-considered and applied to other organisms (Bosco and Haber 1998). Below is a review of the evidence supporting a replicative recombination mechanism in *E. coli*.

First evidence for replicative recombination in *E. coli*

The postulated involvement of DNA replication in recombination has a long history (see above, and referenced in Kuzminov and Stahl 1999); however the first correlative evidence, connecting the two processes in *E. coli*, did not emerge until much later (Siegel 1974), and was largely ignored. Siegel developed an elegant assay, with 1974 technology, to detect new DNA synthesis within fragments of λ recombinant progeny. He used λ as the DNA substrate for RecBCD recombination. Phages, defective for their own recombination systems and carrying a recombination hotspot for the RecBCD pathway, Chi, were infected into *E. coli* under conditions that allowed for little DNA replication. Recombinant phage were isolated and fragments of their DNA was examined for incorporation of $^{32}PO_4$. He found that DNA fragments containing the Chi sequence also had the most amount of radioactive label, thus correlating DNA replication and recombination on the same DNA molecule for the first time. He proposed a replicative recombination model that was truly revolutionary for its time: perhaps too advanced to be considered strongly by his colleagues at that time. Here is a direct quote from the paper:

> "Compatible with the data reported here is the hypothesis that Rec recombination occurring in the absence of normal

> DNA duplication creates an "origin" for DNA synthesis at the site of the recombination event. Synthesis proceeds in either or both directions and terminates after a variable distance ...."

The hypothesis that DNA recombination intermediates can serve as replication "origins" was supported by these data for the first time in *E. coli*. Unfortunately, this work was rarely cited and the importance of this hypothesis was not investigated for years to come. Later, the replicative recombination hypothesis re-emerged (see Smith 1991) as new evidence suggested the occurrence of this type of replication in *E. coli* (e.g. Kogoma 1997).

Subsequent indirect evidence for the involvement of DNA replication in recombination in *E. coli*

In recent years, a large body of work from different labs has suggested the involvement of DNA replication in recombination in *E. coli*. This is reviewed in detail in Chapter 2; however, a brief summary of some of the important results and the roles of implicated recombination and replication proteins will facilitate the understanding of Chapters 2 and 3 of the thesis.

Recombination-dependent stationary phase mutation

The discovery of a recombination-dependent mutational mechanism operating in stationary phase *E. coli* has provoked models of RecBCD-mediated replicative recombination (reviewed in Rosenberg et al. 1998; Lombardo and Rosenberg 1999). In this system, homologous recombination proteins (such as RecA, RecBC, RuvABC) and DNA Polymerase III are required for the mutational mechanism. The data can be easily explained by models in which recombination intermediates, formed via the RecABCD system, initiate DNA replication that leads to polymerase errors and mutations (see Rosenberg et al. 1998; Lombardo and Rosenberg 1999). Because in this assay mutations are genetically selected, the direct demonstration of recombinant DNA that has also experienced new DNA synthesis has not been made. Data from other labs have also provided suggestive evidence for the interdependence of DNA replication and recombination in *E. coli* (Courcelle et al. 1997; Courcelle and Hanawalt 1999; Kuzminov and Stahl 1999); however, the direct demonstration of replicated recombinants still remains. In Chapter 2, I show direct physical evidence of recombinant molecules that have also experienced new DNA synthesis required for their formation in wild-type *E. coli*. I show that this type of replicative recombination occurs normally and is responsible for roughly half of all RecBCD-mediated events.

Over-representation and asymmetric distribution of Chi sites in the *E. coli* genome

As discussed previously (see above), Chi sites are special cis-acting octameric DNA sequences that enhance recombination only in the RecBCD system. These sites were initially discovered by Stahl's group as mutations in the sequence of λ genome that confer better growth of the phage in *E. coli* (Lam et al. 1974). Further characterization revealed Chi is recognized by RecBCD only if it is encountered from the 3' GG side. The Chi-RecBCD interaction modifies the biochemical activity of the enzyme such that more substrate for recombination (ss DNA) is generated for RecA-mediated strand invasion and heteroduplex formation (see Figure 1-2 for a model for RecBCD-mediated recombination).

Interestingly, Chi is over-represented by roughly 4-8 fold in the *E. coli* genome than would be expected by chance alone. Furthermore, these sites are distributed asymmetrically in the *E. coli* chromosome (Blattner et al. 1997):

approximately two thirds of all Chi sites are situated such that they face the origin of replication *oriC* (reviewed in Kowalczykowski 2000). These findings can be explained neatly by a hypothesis that Chi promotes the repair of collapsed replication forks in *E. coli* (Kuzminov 1995). For example, if a break in the template occurs, forming a DSB, the ensuing re-attachment of the broken arm with the sister duplex can occur by a RecBCD-Chi-dependent mechanism (Kuzminov 1995). This is suggestive of the existence of a RecBCD-mediated recombinational repair pathway used to restart collapsed replication forks in *E. coli*, connecting the processes of DNA replication and recombination.

Stable DNA replication (SDR)

One of the most provoking pieces of evidence supporting the interplay between DNA replication and recombination comes from the work of the late Tokyo Kogoma. He was the first to demonstrate the existence of a DNA replication mechanism that operates independently of protein synthesis and the origin of replication (*oriC*) but requires DNA recombination proteins. His initial discovery, that wild-type levels of DNA replication can occur in cells defective for *oriC*, was made in 1970; however, his work was not considered a general mechanism for DNA replication or recombination by other researchers until much later (Kogoma and Lark 1970). This type of replication is known as stable DNA replication (or SDR), and occurs only under special conditions (e.g. during the SOS response to DNA damage) or in special mutant *E. coli* cells (*rnhA* mutant cells) (reviewed in Kogoma 1997). The involvement of recombination proteins in this process was shown genetically: some of the proteins required for RecBCD-mediated recombination and DNA replication have also been shown to be required for this process. Kogoma extended these observations to propose a direct link between replication and recombination in *E. coli*. However, the direct demonstration of recombinant DNA molecules that have also experienced DNA replication was not made.

Of interest, two proteins are required for this type of replication: RecA, the *E. coli* strand-exchange protein (see above and Roca and Cox 1997), and PriA, the replication primosome assembly protein (reviewed in Marians 2000; Sandler and Marians 2000). A primosome is a group of enzymes that can unwind duplex DNA and synthesize short oligoribonucleotides primers on the DNA template, required for DNA replication. The data genetically connect proteins previously characterized for their role in recombination to the DNA replication process. The discovery of SDR and the genetic characterization of the proteins involved in this process prompted research in the field and provoked models connecting DNA replication and recombination in *E. coli*, backed by experimental data (e.g. Kogoma 1997).

PriA protein

The biochemistry and genetics of the PriA protein has been studied extensively. Much of the data suggest a role for PriA-mediated DNA replication in the formation of recombinant DNA (Marians 2000). Again, the direct demonstration of this connection (the isolation of recombinant DNA that has replicated) has not been made.

PriA is multifunctional with ATPase, helicase and translocase activities, and can direct the assembly of primosomes on DNA intermediates (reviewed in Marians 2000; Sandler and Marians 2000). PriA appears to be required for various cellular activities. Cells defective for PriA are sickly, exhibit low viability and show a plethora of other phenotypes, including sensitivity to DNA damaging agents (such as UV), constitutive induction of the SOS response, a two-third decrease in recombination, and are defective in SDR and re-start of collapsed replication forks. The replication and recombination defects seen in *priA* mutant cells suggest an interconnection between the two; however, it's not known whether the defects in the replication-promoting activities of PriA are directly involved in the decrease in recombination seen in these cells.

Interestingly, PriA-mediated DNA replication may play a role in the processing of HJs in vivo. PriA has a DNA binding activity to branched DNA structures (e.g. D-loops), similar to Holliday structures, and a proposed intermediate of replicative recombination (Kogoma 1997). PriA has been shown to compete with HJ processing protein RecG for binding to branched DNA intermediates indirectly in vivo (Al-Deib et al. 1996) and directly in vitro (McGlynn et al. 1997). The in vivo study revealed that suppressers of *recG* mutation, which display reduced recombination and DNA repair, were found in the helicase domain of PriA protein. Because PriA was shown to have a 3' to 5' DNA helicase activity, it was proposed that this activity of PriA inhibits recombination and that this effect is countered by RecG in wild-type cells. Further support to this model came with the biochemical characterization of DNA substrates for these two proteins: PriA and RecG were shown to compete for binding to a set of branched DNA structures (e.g. D-loops) in vitro. Taken together, these data suggest an intimate interplay between DNA replication and recombination, even at the level of processing of recombination intermediates. In Chapter 2, we show that in the absence of Ruv and RecG, DNA replication is required for recombination, effectively substituting for HJ processing in vivo. In Chapter 3, we present data that suggest two distinct recombination intermediates occur in vivo, one of which is specifically processed by DNA replication, independently of Ruv or RecG proteins.

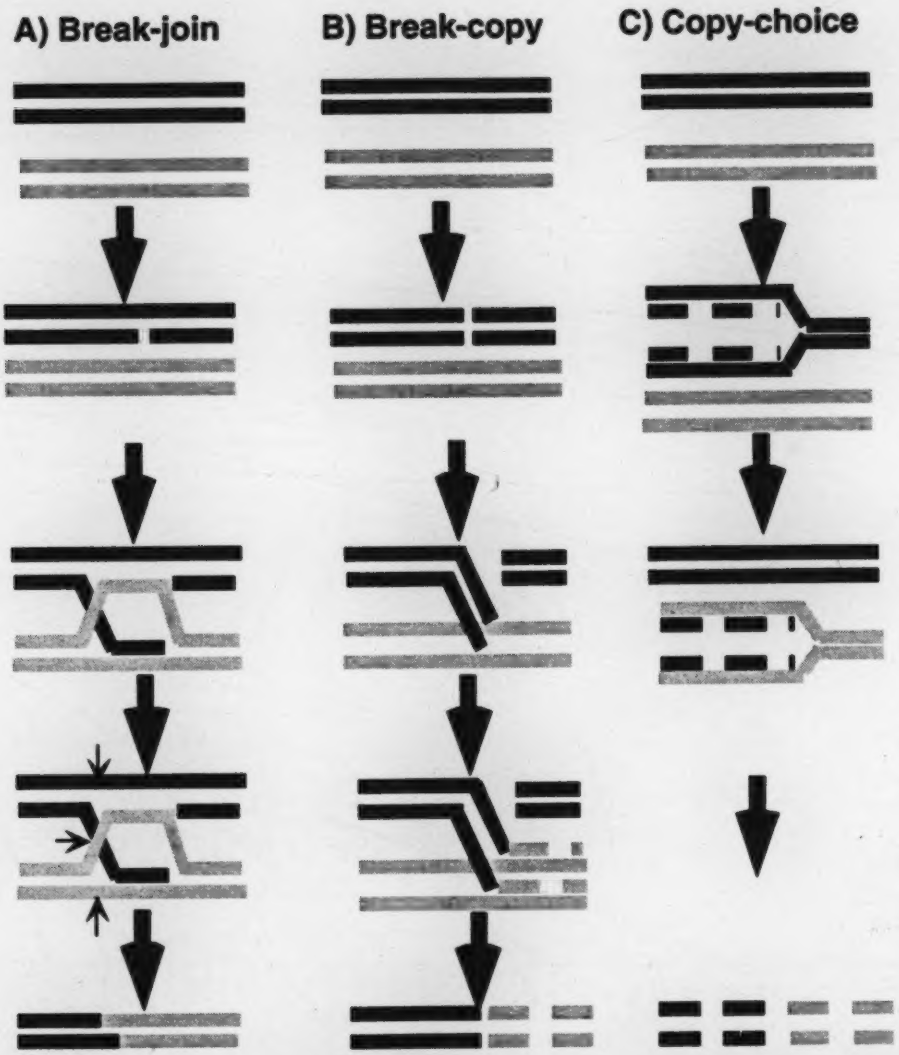

Figure 1-1. Three general schemes for recombination. Please note that these are modern re-interpretations of the original models. Solid lines represent old parental DNA; dashed lines represent newly synthesized DNA. A) In break-join models, recombination occurs by cutting and re-ligation of homologous molecules, without the involvement of any DNA synthesis. The thin arrow represents the site for an endonucleolytic cleavage of the duplex molecule. B) In break-copy models, recombination intermediates initiate replication such that the recombinant molecule is a hybrid of old and newly synthesized DNA. C) In copy choice models, recombination occurs when the advancing replication fork switches templates and copies information from the homologous partner. In this scheme the recombinant molecule is made entirely from newly synthesized DNA.

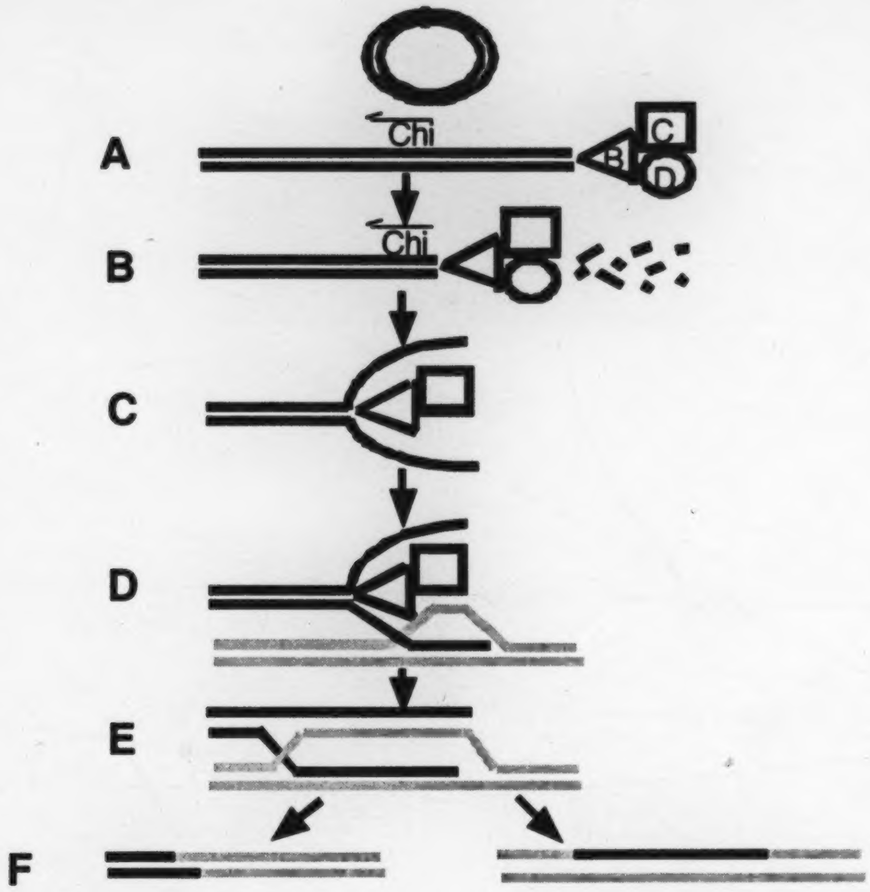

Figure 1-2. A model for RecBCD-mediated recombination (adapted from Rosenberg and Hastings 1991) where both 3' and 5'-ending strands are recombinogenic. The components of RecBCD enzyme are represented as triangle, square and circle. Upon an encounter with Chi, it is proposed that the regulatory subunit of the enzyme, RecD, is ejected, thereby modifying the activity of RecBCD from a DNA exonuclease to a DNA helicase. The helicase activity of RecBCD is proposed to generate ssDNA which is the substrate for RecA enzyme.

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Chapter 2[*]

Double-strand break-repair recombination in *E. coli*: physical evidence for a DNA replication mechanism in vivo

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Introduction

DNA double-strand breaks (DSBs) are common lesions that occur in all cells. They result from DNA damage, from processing of arrested replication forks (Seigneur et al. 1998) and are hypothesized to occur as normal intermediates in DNA replication, (e.g. Skalka 1974; Kuzminov 1995). Because DSB accumulation is toxic to cells, multiple mechanisms have evolved for their repair. Homologous recombination may be the exclusive mechanism for DSB-repair (DSBR) in *Escherichia coli*, is the dominant mechanism in some eukaryotes including baker's yeast, and is one alternative in mammals including humans (e.g. Haber 1999). Simple ligation of DNA ends (non-homologous end-joining), is a major alternative repair route in mammals which often results in loss of genetic material and gross chromosome changes (Tsukamoto and Ikeda 1998; Haber 1999). DSBR via recombination is conserved in evolution, as are its important proteins, and it is required for the normal functions of cells (for review, see Kanaar and Hoeijmakers 1998; Haber 1999). Aberrant DSBR could underlie the excessive recombination linked to phenotypes of genetic instability, premature aging, and cancer (e.g. Ellis et al. 1995; Yu et al. 1996).

In addition to its roles in the maintenance of genomic stability, homologous recombination creates new cellular and organismal combination of alleles, and ensures proper segregation of chromosomes during meiosis. In *E. coli*, the RecBCD recombination system provides nearly all DSBR (Kowalczykowski et al. 1994; Myers and Stahl 1994) and catalyzes recombination of the linear DNA intermediates in conjugation and phage-mediated transduction, two important avenues of genetic exchange between bacterial cells (Clark and Sandler 1994; Lloyd and Low 1996; Rosenberg and Motamedi 1999).

Double-strand break-repair is also the major sexual recombination route in yeast meiosis (Haber 1998; Smith and Nicolas 1998).

Possible styles of recombination can be defined based on the proposed involvement of DNA replication (Meselson and Weigle 1961): Break-join recombination models use no replication. Parental DNAs are cut and rejoined, producing recombinant molecules made entirely of parental DNA. Break-copy models use a fragment from one parental molecule to prime replication from a homolog, thereby producing recombinant molecules with DNA material from one parent joined to new DNA carrying information from the other. A paradox for the RecBCD system is that the only direct physical evidence bearing on whether recombined DNA is replicated has demonstrated clearly the existence of break-join recombinants (see below). However there is mounting suggestive, but indirect, evidence that would be unified by the existence of a break-copy pathway.

The direct evidence for break-join recombination was obtained using techniques in which phage lambda (λ) DNA is used as a substrate for the bacterial recombination system (Meselson and Weigle 1961; Meselson 1964; Stahl and Stahl 1971; McMilin and Russo 1972). [λ lacking its own recombination genes recombines exclusively via a RecBCD-dependent mechanism (Lam et al. 1974; Henderson and Weil 1975). λ is the molecule with which the RecBCD system's recombination hotspot sequence Chi was discovered.] Using density-labeled λ (^{13}C and ^{15}N) that were allowed to recombine in unlabeled *E. coli*, these groups separated unreplicated from replicated λ progeny in cesium formate equilibrium density gradients. They determined that recombinants were present among fully unreplicated molecules, and could even occur under a full block to replication, thereby providing direct physical evidence for RecBCD-mediated break-join recombination in *E. coli* (McMilin and Russo 1972; McMilin et al. 1974).

Although break-copy mechanisms were not excluded (see Siegel 1974), break-join was considered to be the major route for RecBCD-mediated recombination (e.g., Thaler and Stahl 1988; West 1992; Kowalczykowski et al. 1994). The apparent dominance of break-join was bolstered by the discoveries of endonucleases specific for the strand-exchange junctions [such as Holliday junctions (HJs)] which connect recombining molecules (Kemper et al. 1984; Connolly et al. 1991; Sharples et al. 1998) and by the demonstration of a requirement for such enzymes for conjugational and transductional recombination in *E. coli* (Lloyd 1991). Such endonucleases are expected to be required for completion of break-join events, for example, for breaking the molecule indicated by the open arrow in Figure 2-1.

More recently, good arguments for why replication should be a possible consequence of RecBCD-mediated recombination and DSBR in *E. coli* have been advanced (Smith 1991). However, much of the evidence in apparent support of break-copy models has been obtained under special circumstances, and all of it to date has been indirect (for review, see Discussion) in that replication and recombination were not demonstrated to have occurred in the same DNA molecules.

Here, we present physical evidence that replicational recombination is a major route to DSBR in *E. coli*, in addition to the established break-join mechanism. We used phage λ DNA (lacking the λ recombination functions) as the substrate for RecBCD-mediated recombination because well-established, sensitive methods allow DNA labeling and physical detection of new DNA. λ has the advantages that all RecBCD-mediated DSBR uses a known, defined break-site - the packaging origin, *cos*, which is cleaved during DNA packaging

(Kobayashi et al. 1982; Kobayashi et al. 1983; Thaler and Stahl 1988) - and that recombinant DNAs are packaged into phage particles selectively. Using physical analysis of the recombined DNAs, we find that about half of all RecBCD-mediated recombination of λ DNA occurs by a break-join mechanism. We show that the Holliday junction processing proteins of *E. coli* are required for that mechanism, whereas the major replicative polymerase, DNA Polymerase III (Pol III), is not. We report the discovery of a second RecBCD-mediated recombination mechanism that is independent of the Holliday junction processing proteins, and requires DNA Pol III. This recombination occurs only when DNA replication is permitted, and produces recombinant molecules that all contain some newly synthesized DNA, demonstrating a direct physical association of recombination with replication in the same DNA molecules. The extent of the new DNA synthesis is compatible with break-copy models (alternative discussed below). This replicational recombination mechanism accounts for about half of all RecBCD-mediated recombination of λ DNA.

The results demonstrate a replicational recombination route in the RecBCD system of DSBR recombination in *E. coli*, showing the existence of the replicated recombinant molecules directly. We also show that the two mechanisms, replicational and break-join recombination, can be separated.

Results

Strategy for blocking break-join recombination

We sought to determine whether a replicational mechanism of recombination occurs in the RecBCD system, in addition to the established break-join process. Because any putative replicational recombination might be easier to detect in the absence of break-join events, we attempted to block break-join recombination. We hypothesized that break-join recombination might have a unique requirement for the proteins that process strand-exchange recombination intermediates, or Holliday junctions, in *E. coli*. In Figure 2-1, note that break-join recombination actually requires two DSBs: one to initiate attachment of the broken molecule to a homolog; and another (thin arrow) to break the homolog so that it can ligate with the DNA fragment that invaded it. This second break occurs in a strand-exchange junction (Figure 2-1). A Holliday junction cleaving protein, such as the *E. coli* RuvC endonuclease (Connolly et al. 1991), might be expected to make this second break in vivo. Because the *E. coli* RecBCD system uses either of two systems for processing strand-exchange intermediates, the RuvABC or the RecG systems (Lloyd 1991), for processing branched intermediates, we attempted to detect RecBCD-mediated recombination of phage λ DNA in the absence of both systems, in *ruv recG* double mutant cells. In this paper, all the possible branched intermediates will be referred to as HJ for Holliday junctions and other branched intermediates.

λ *red gam* mutants form plaques on *E. coli ruv recG* strains

One measure of λ recombination in the RecBCD system is the ability of λ recombination-defective strains (λ *red gam*) to form plaques on RecBCD$^+$ *E. coli*, (for review, see Smith and Stahl 1985) In RecBCD$^+$ *E. coli*, λ *red gam* rolling circle replication does not occur detectably because RecBCD destroys rolling circles. [Wild-type λ makes Gam protein, a specific inhibitor of RecBCD, preventing the degradation of rolling circle intermediates. In the absence of λ recombination systems, Int and Red, the *E. coli* RecBCD pathway operates as the only means for recombining λ DNA.] The monomeric λ chromosomes produced by bidirectional (theta) replication must recombine to form packagable substrates [dimers and multimers are packagable whereas monomers are not (Feiss and Becker 1983). Because only the host RecBCD pathway is available for recombination, λ *red gam* cannot form plaques on cells that are recombination-defective such as *recA* null mutant strains. The data in Table 2-1 reveal that unlike *recA* strains, *ruvA recG* and *ruvC recG* double mutant cells allow plaque formation of three different λ *red gam* strains. This is observed for *ruv recG* combinations constructed in two different *E. coli* genetic backgrounds (Table 2-1; Materials and Methods). Plaques were about the same size as those on isogenic *rec*$^+$ parents, and did not form on *recA* control strains (not shown). These data suggest that, unlike *recA* strains, *ruv recG* double mutants allow RecBCD-mediated recombination of phage λ DNA. To be sure that this plaque formation reflected recombination-proficiency, we measured the frequencies of RecBCD-mediated λ recombination in the absence of Ruv and RecG functions using a quantitative assay.

Assays for the frequency of RecBCD-mediated recombination

A standard assay was used to measure the frequency of RecBCD-mediated recombination of λ DNA (Figure 2-2A). As with the experiments reported above (and in all experiments in this paper) the λ used are *red gam* so that recombination is exclusively via the host RecBCD system. Also, as described above, this means that all progeny must contain recombinant chromosomes (whether these are detectably recombinant, resulting from recombination events between phages of two different genotype, or occurred between phages of the same genotype). To measure the frequency of homologous recombination in the face of this requirement for recombination, one can provide an alternative route to dimerization (and packaging) so that any homologous recombination events are gratuitous and quantifiable. In the assay used here (modified from Thaler et al. 1989; Razavy et al. 1996), dimerization is achieved via the λ Int system of site-specific recombination, and gratuitous homologous crossovers (splices) are measured only from among the site-specific recombinants. This assay is sensitive and the results correlate well with standard P1 transductional recombination assays (Razavy et al. 1996; Razavy 1997).

In Figure 2-2A, note that site-specific Int-dependent recombination occurs between two half *att* sites of the recombining λ molecules. These sites have too little sequence identity for homologous recombination. One parent is deleted from the *att* site leftward (Δ), whereas the other carries a deletion-substitution (*bio1*) from *att* rightward. These net deletions decrease the size of the λ chromosome, but do not alter the size of the phage capsid, so that phages carrying the Int-mediated recombinant with no net deletions are denser than either parent (more DNA in the same size capsid). The denser site-specific recombinant can be separated from both single deletion (parental) phages in a cesium formate equilibrium density gradient (Razavy et al. 1996) (Figure 2-2B), and homologous exchanges quantified from among them.

RecBCD-mediated λ recombination is equally efficient in *rec*$^+$ and *ruv recG* cells

The amount of λ recombination in *ruvC recG* cells was quantified using the assay described above using a set of phages illustrated in Figure 2-2A. In Figure 2-3 (left panel), results from three independent experiments performed in *rec*$^+$ and *ruvC recG* strains are summarized in the *nin*$^+$ panel (the significance of *nin* is discussed below). We observed no significant difference in the percentage of λ recombinants between crosses performed in *rec*$^+$ and isogenic *ruvC recG* cells.

The λ *nin* region encodes a function(s) responsible for approximately half the recombination in *ruvC recG* cells

A possible reason for the high efficiency of λ recombination in *ruv recG* cells could be that a λ-encoded Holliday junction resolvase substitutes for the *E. coli* Ruv or RecG proteins. The nonessential λ *nin* region encodes analogs of *E. coli* recombination proteins including a demonstrated resolvase, Rap (Sharples et al. 1998). We performed similar λ crosses with phages deleted for this region. The results of three independent sets of experiments are displayed in Figure 2-3 (Δ*nin* panel). We found that when the *nin* region is deleted, λ recombination is decreased by approximately half in *ruvC recG* cells compared with the *rec*$^+$ controls. This supports the hypothesis that a λ encoded function can resolve recombination intermediates in vivo. However, it does not address how the remaining half of λ recombination works in *ruv recG* cells. To explore the mechanism of recombination in the absence of these known HJ processing proteins, and more specifically, to test whether it is replicational, all of the remaining experiments presented were conducted using λ phage carrying the deletion Δ*nin5*.

Recombination in the absence of RuvC, RecG, and λ *nin* is not catalyzed by *E. coli* defective prophage-encoded recombination functions

Most *E. coli* K-12 strains harbor a number of defective prophages, which are thought to have been acquired following lysogeny and subsequent loss of phage functions required for lytic growth. Defective prophages often are not expressed and perform no function in wild-type *E. coli*, but can sometimes become activated following infection by a lambdoid phage (for review, see Campbell 1996). The *E. coli* strain used in the experiments above carries at least two defective prophages with known recombination functions, *rac* and *qsr*. The *rac* prophage carries the *recE* and *recT* genes which, when activated, can perform RecABC-independent recombination (Clark and Sandler 1994). The *qsr* prophage carries the *rusA* gene, which is not normally expressed, but once activated, can resolve HJs in a manner similar to RuvC endonuclease, and can substitute for Ruv proteins *in vivo* (Mahdi et al. 1996). To test the possibility that either RecET or RusA function substitutes for the *E. coli* Ruv and RecG proteins in λ recombination, we performed λ crosses (with λ Δ*nin*, as described above, Figure 2-2) in cells that carry either a deletion for the *rac* prophage or for the *rusA* gene. We observed no difference in λ recombination frequency in *ruvC recG* and *ruvC recG* Δ*rac* or *ruvC recG* Δ*rusA* cells (Table 2-2). Therefore, functions from these prophages are not responsible for the recombination in λ Δ*nin ruv recG* experiments.

Chi stimulates recombination normally in the absence of *nin*, RuvC and RecG: the RecBCD system performs apparent resolvase-independent recombination

The Chi site (5'GCTGGTGG) promotes RecBCD-mediated recombination and DSBR specifically (Kowalczykowski et al. 1994; Myers and Stahl 1994; Eggleston and West 1996). It is the DNA recognition sequence of the RecBCD enzyme (Ponticelli et al. 1985), and promotes RecBCD-mediated recombination in its own vicinity, acting as a recombination hotspot. To test whether the apparently resolvase-independent recombination of λ in *ruvC recG* cells is normal RecBCD-mediated recombination, we tested whether Chi stimulates recombination normally in the absence of RuvC and RecG. The frequency of recombination was quantified from λ crosses performed in parallel with Chi^{+} and Chi^{o} phages in *rec*$^{+}$ and *ruvC recG* cells. The data in Figure 2-3 ($\mathrm{Chi}^{+/o}$ Δ*nin* panels) show that Chi promotes recombination as well in the absence of RuvC and RecG as in their presence. Chi activity (recombination frequency in the Chi^{+} cross/ recombination frequency in the Chi° cross) was 3.3-, and 3.3-fold in *rec*$^{+}$ (experiments 1 and 2) and 3.8- and 3.9-fold in *ruv recG* cells (experiments 1 and 2 respectively). [These are typical Chi values for recombination in the large DNA interval measured (Razavy et al. 1996)]. We conclude that Chi-stimulates RecBCD-mediated recombination normally in the absence of the known HJ processing proteins. This RecBCD-mediated recombination is replicational, as shown below.

RecBCD-mediated recombination in *ruvC recG* cells is replication- dependent and requires DNA polymerase III

We hypothesized that replication may help to resolve recombination intermediates, perhaps by making endonucleolytic cleavage unnecessary, as illustrated for break-copy recombination in Figure 2-1 (see also Morgan and Severini 1990). If this were the case, the recombination in the absence of known HJ processing proteins would be replication-dependent. We therefore assayed λ recombination in the absence of the known resolvases (RuvC, RecG and *nin*-encoded Rap) and DNA replication. DNA replication was blocked using a temperature-sensitive allele of *dnaE* encoding the core enzyme of DNA Pol III, the major replicative polymerase of *E. coli* (*dnaEts486*; see Materials and Methods), and shifting the cells to restrictive temperature for the λ infections. Because the Int site-specific recombination system (Figures 2-2 and 2-3) is temperature-sensitive, and therefore inappropriate, we used a different assay for recombination-proficiency in these replication-blocked experiments (modified from Stahl et al. 1972b, see Materials and Methods). As discussed, λ progeny formation requires recombination. Because λ DNA multimers are required for packaging in RecBCD$^+$ cells, the only route to multimerization, and therefore progeny formation of these Int$^-$ phage, is via homologous recombination of monomers. Therefore, λ infections yield phage progeny only if cells are recombination-proficient. Thus, if replication is required for recombination when the resolvases are absent, no progeny should be detected in the absence of replication in *ruv recG* cells.

Int$^-$ λ phages density labeled with ^{13}C and ^{15}N were infected into unlabeled *E. coli* cells that carry the *dnaEts486* allele. A complete replication block was achieved by performing the experiments at high temperature (43.5°, Figure 2-4; Methods). Any new DNA synthesis would incorporate light nucleotides. This can be detected in a cesium formate density gradient of the phage progeny (Figure 2-4).

In Figure 2-4A, note the two peaks of phage that emerge from infection of *rec*$^+$ cells. The denser peak represents phage that possess heavy protein coats in addition to their fully heavy (HH) DNA. These are unadsorbed phage that did not enter the light *E. coli* and are not part of the progeny. The less dense peak represents phage with light capsids and unreplicated (HH) DNA. These are phage progeny resulting from break-join recombination events (this point is confirmed below, and in Figure 2-6, see below). Because these phage have no Int (site-specific recombination) system operative (Materials and Methods) they are inferred to have resulted from RecBCD-mediated break-join recombination. This is confirmed in a parallel infection of *recA* recombination-defective cells (Figure 2-4C), in which few or no λ progeny are produced (because recombination is required for packaging). The absence of lighter peaks confirms that the replication block was complete.

Importantly, we recovered few or no phage progeny from *ruvC recG* cells when replication was fully blocked (Figure 4B). These data demonstrate that recombination in *ruvC recG* cells requires DNA replication. Because DNA replication was blocked by use of *dnaEts*, a mutation of the structural gene encoding Pol III, the data also identify DNA Pol III as the polymerase required for this replication. Thus, the data imply that recombination in the absence of RuvC and RecG is replicational. We hypothesize that unresolved recombination intermediates in the *ruv recG* cells initiate replication forks, as in break-copy models (Figure 2-1, Figure 2-5) and that DNA replication to the end of the chromosome can produce recombinant molecules.

Physical evidence for a break-copy mechanism

Figure 2-5 outlines some specific predictions of break-copy recombination models. If recombination occurs between density labeled DNAs (thick solid lines, Figure 2-5B-D) in unlabeled cells, then break-copy recombinants that occur in the center of the chromosome should contain both heavy, unreplicated parental DNA (solid lines Figure 2-5B) and newly-replicated, light DNA (dashed lines Figure 2-5B). End recombinants formed by break-copy could contain almost all heavy DNA with just a little new, light DNA (Figure 2-5C). This contrasts with the prediction for break-join recombination, in which even central recombinants should be fully unreplicated, composed of fully heavy (HH) DNA (Figure 2-5D).

Our results above suggested that in *rec*$^+$, break-join (HH central recombinants) should be present (and break-copy recombinants might too), but that in *ruv recG*, there would be no fully HH central recombinants. We tested these predictions using a λ recombination assay (Meselson 1964; Stahl et al. 1972a; Sawitzke and Stahl 1997) in which density-labeled phages recombine in the presence of light isotopes in *E. coli* in which a small amount of DNA replication is permitted. The partial replication-block was achieved as described (Sawitzke and Stahl 1997) with the addition that a special allele of the *E. coli dnaB* replication helicase gene was used (*grpD55*) which blocks use of the λ replication origin by DnaB, but allows normal *E. coli* replication (Bull and Hayes 1996) (Materials and Methods). The phages (Sawitzke and Stahl 1997) are marked such that recombination events occurring in the center of the chromosome (between the *J* and *cI* genes, Figure 2-6A) can be measured separately from recombination events occurring at the right end of λ chromosome (between the *cI* and *S* genes, Figure 2-6A). The λ Int (site-specific) system is inactivated by mutation such that only RecBCD-mediated homologous recombinants are measured (Sawitzke and Stahl 1997) (Materials and Methods).

Progeny phage can be separated physically from (parental) unadsorbed phage based on their densities. The unadsorbed phage occupy the densest peak of cesium formate density gradients (Figure 2-6B and C). The cross progenies are further separated based on the extent of DNA synthesis in each packaged DNA molecule. Mostly or completely unreplicated (heavy-heavy, HH), and replicated (heavy-light, HL, and light-light, LL) progeny are distinguished physically in this assay (Figure 2-6B and C). Intermediate densities are also seen. The amount of central and right end recombination is assayed for each gradient fraction (Figure 2-6A, Materials and Methods).

Representative data presented in Figure 2-6B and C (and numerous independent experiments that repeated these results) allow the following conclusions:

(i) The HH peak from the *rec*$^+$ infection contains central recombinants (Figure 2-6B, filled circles). We conclude that these have arisen via a break-join mechanism, without extensive synthesis of DNA.

Note that the number of central recombinants (filled circles) exceeds the number of end recombinants (open circles) in the HH peak in *rec*$^+$ (Figure 2-6B). This presumably reflects the larger size of the central interval (between 18-22 kb) than of the end interval (4.8 kb). [We express the central interval as a range because the exact position of the *Jts* allele is not known; 18 and 22 kb are the distances between the ends of the *J* gene and the *cI* marker (Daniels et al. 1983)].

(ii) There are essentially no central recombinants (filled circles) in the heaviest fractions of the HH peak in the *ruvC recG* infection (Figure 2-6C). Note that in *ruvC recG*, there are *more* end (open circles) than central recombinants in the HH peak (Figure 2-6C, fractions 24, 25). These data indicate that break-join recombination yielding HH central recombinants does not occur appreciably in the absence of RuvC and RecG. This supports the conclusions from results shown in Figure 2-4, in which no recombinant progeny were produced when replication was completely blocked in a *ruvC recG* strain. The presence of even a small number of end recombinants in the *ruvC recG* HH peak (Figure 2-6C) may seem inconsistent with the absence of any recombinants at all in *ruv recG* cells when replication is completely blocked (Figure 2-4). We suggest that the end recombinants in the HH peak have probably experienced a small amount of replication, but not enough to separate them from the HH peak (see Figure 2-5C).

(iii) The central recombinants in *ruvC recG* , which are absent from the HH peak, are seen here in the HL peak (Figure 2-6C). Note that almost all of the central recombinants in *ruvC recG* are in the HL peak. This excess of central recombinants in the HL peak is expected if the central recombinants are formed by replication, suggesting that recombination reactions initiated at the center are completed by replicating out to the end of the chromosome (Figure 2-5B). This result supports break-copy models (see Figure 2-5, other possibilities discussed below) and demonstrates directly that the recombinant molecules formed in the absence of Ruv and RecG are replicated.

Physical evidence for break-copy and break-join recombination pathways in *rec*+ cells

As discussed above, accumulation of central recombinants in the HL peak of *ruv*C *rec*G cross is expected if replication is used to form central recombinants. Informatively, we also see this accumulation of central recombinants in the HL peak of *rec*+ crosses (Figure 2-6B, compare the ratio of central/end recombinants in the HL peak with the HH peak). This is the first direct demonstration of replicative recombination in the RecBCD pathway in *rec*+ cells, *i.e.* the replicated DNA is present in the same DNA molecules that have recombined (other evidence reviewed below). Previous direct evidence bore on the existence of the break-join mechanism only (McMilin and Russo 1972; Lam et al. 1974, also Figure 2-6B, HH peak). These data show that a significant fraction of recombination in wild-type *E. coli* occurs via a replicative mechanism even when Ruv and RecG functions are present.

The ratio of central/end recombinants in the HL peak is 5.3, or about twice that seen in the HH peak (2.5), thus implying that about half the recombination in *rec*+ is replicative. The rough equality of replicative and break-join recombination was also inferred from the observation that, in *ruv recG* cells, recombination frequency drops to half that seen in *rec*+ (Figure 2-3, Δ*nin*) in which no break-join events can occur (Figure 2-4, Figure 2-6C), and all recombination is replication-dependent (Figure 2-4).

Estimation of the amount of DNA replication associated with recombination

A rough estimation of the amount of newly synthesized DNA associated with recombination in the cross displayed in Figure 2-6C can be made as follows. The number of fractions between the fully heavy and fully light shows that each fraction accounts for a change of about 8.3 percent in the proportion of the DNA that is heavy or light. If the segregation of old and newly synthesized strands following recombination is conservative (see Figure 2-5), then a change of 1 fraction also represents a change of 8.3 percent of the length of the λ genome from heavy to light. For *ruvC recG*, the fractions with an excess of central recombinants (27-32, Figure 2-6C) correspond to 17 to 58 percent of the genome being new (the most abundant fraction having about 50% new DNA). This is a remarkable correspondence with the distance of the central recombination events (recombination between *J* and *c*I) to the λ right end. *J* is between 59 and 66 percent of the λ genome from the right end (the position of the *Jts* marker is unknown), whereas *c*I is 17 percent from the right end. This observation is compatible with break-copy models with a conservative segregation of new strands as proposed in Figure 2-5. Semiconservative segregation would produce half as much new DNA. These data show that not only is new DNA synthesis present directly in the same DNA molecules that recombined, but also that the amount of synthesis corresponds to that expected from the cross-over point to the end of the chromosome (Figure 2-5B) as in break-copy models (alternatives discussed below).

Absence of RuvC and RecG promotes replication of λ

An unexpected but highly informative result was seen in the experiments performed in parallel, shown in Figure 2-6B and C. Although the experiment was performed under the same conditions in *rec*$^+$ and *ruvC recG* cells, we observed approximately 135 times more phage with replicated DNA when the *E. coli* Ruv and RecG resolution systems were absent. This was calculated by dividing the area under the LL peak of the *ruvC recG* graph with the LL peak for *rec*$^+$. (This difference is especially apparent in the LL peaks of the *rec*$^+$ and *ruvC recG* gradients shown in Figure 2-6, in which the titer of LL phage is 8.7×10^3 and 1.2×10^6 for *rec*$^+$ and *ruvC recG*, respectively. We excluded the HL peaks from these calculations because in *rec*$^+$, some HL recombinants will be break-join events between HH and light molecules.) This result was repeated in two additional experiments in which the extent of phage with replicated DNA in *ruvC recG* was 108 times and 74 times greater than in *rec*$^+$ cells. These data suggest that strand-exchange (HJ) intermediates, which accumulate in the absence of Ruv and RecG HJ processing proteins, promote replication.

Discussion

The data shown here demonstrate the following:

(i) RecBCD-mediated λ recombination in the absence of the *E. coli* Ruv and RecG HJ resolution systems is dependent on either a *nin*-encoded function(s) or DNA replication. Each accounts for approximately half of the total recombination in these cells (Figures 2-3, 2-4, 2-6A, see above). The *nin* encoded function responsible has not been identified but is likely to be the Rap Holliday junction resolvase (Sharples et al. 1998), which facilitates some kinds of recombination events in vivo (Hollifield et al. 1987; Stahl et al. 1995).

(ii) λ recombination in the absence of the known HJ resolution systems requires the major replicative polymerase, DNA Pol III (Figure 2-4).

(iii) Direct physical analysis of recombined DNA for incorporation of new (light) isotopes revealed that break-join recombination occurs in wild-type cells (Figures 2-4, 2-6, McMilin and Russo 1972; Stahl et al. 1972a; McMilin et al. 1974) and absolutely requires Holliday junction processing proteins such as Ruv, RecG, or the *nin* function (Figures 2-4, 2-6).

(iv) Both classes of recombination utilize Chi sites efficiently, so we suggest that there are two pathways (and basic mechanisms) of *E. coli* RecBCD-mediated recombination and double-strand break-repair: a break-join pathway that requires Holliday junction resolvases (e.g. see Figure 2-1) and a replicative pathway that can operate independently of resolvases and requires DNA Pol III. We suggest that these are alternative fates of strand-exchange intermediates (e.g. Figure 2-5).

(v) In the absence of resolvases, essentially all of the central recombinants contain newly replicated DNA, indicating that they originated by a replicational recombination mechanism (Figure 2-6C).

(vi) Physical analysis of recombinants in wild-type cells also revealed a substantial fraction of replicational recombination (excess of HL over HH central recombinants) even when the resolvases are present (Figure 2-6B). Therefore we conclude that the replicational recombination pathway is a normal part of RecBCD-mediated λ recombination, not a special mechanism that occurs only in *ruvC recG*-defective cells. In *rec*$^+$ cells, the excess of putative break-copy (HL central) recombination relative to end recombinants in the HL peak is two-fold over that seen in the HH (unreplicated, break-join) peak (Figure 2-6B). This provides independent evidence that about half of RecBCD-dependent DSBR is break-join and the other half replicative.

(vii) The extent of new DNA synthesis in the replicational recombination observed corresponds to the fraction of the λ genome from the crossover point to the λ right end, in support of conservative break-copy models (Figure 2-5, alternatives discussed below).

(viii) DNA replication is promoted dramatically in the absence of RuvC and RecG HJ processing proteins, suggesting that strand-exchange recombination junctions may act as assembly sites for replication forks (this proposal was made previously based on data on recombination-dependent stationary-phase mutation, Harris et al. 1996).

The results summarized above provide a direct demonstration (via detection of replicated recombinant molecules) of a replicational recombination route in the RecBCD system of DSBR recombination in *E. coli*. The data also show that the replicational and break-join mechanisms can be separated: replicational recombination is the only mechanism in *ruv recG* cells (Figures 2-4, 2-6) whereas break-join is the sole route when resolvases are present and replication is blocked (Figure 2-4). These findings will greatly aid further dissections of both RecBCD-mediated DSBR mechanisms.

Previous evidence

Groundbreaking previous work led to the proposal of replicational recombination in *E. coli*. First, the discovery and characterization of a DNA replication mode that is replication origin independent, and recombination protein dependent (stable-DNA replication, or SDR) is most easily understood by the postulate that recombination intermediates initiate replication, as in break-copy models (Kogoma 1997). The evidence is voluminous, important, and highly suggestive, but is indirect. Recombination-related genetic requirements were demonstrated, but DNA molecules that were both recombined and replicated were not. SDR is not a general process because it is seen only in RNaseH-deficient mutants, or during an SOS (DNA damage) response (Kogoma 1997).

SDR-like replication was also observed very recently using phage λ. One λ DNA molecule was shown to be replicated at enhanced levels when a coinfecting λ molecule was linearized ("cut"), and the enhancement required recombination proteins (Kuzminov and Stahl 1999). The results demonstrate replication that is enhanced by recombination proteins and DNA damage. As with SDR, the evidence for association of replication and recombination is indirect for three reasons: (i) the replicated DNA was not shown to have recombined, and recombined DNA showed no evidence of having been replicated (Kuzminov and Stahl 1999); (ii) no requirement for homology between the cut molecule and the replicated molecule was reported; and (iii) all of the recombination proteins implicated (RecA, RecB, RecF) function dually: in recombination; and in induction of the SOS response (Walker 1996). Thus, whether this is SOS-promoted- or recombination-promoted-replication is unknown. Recombination and replication might not have been associated directly in the same DNA molecules.

Second, the existence of a recombination protein-dependent mutation mechanism operating in stationary-phase *E. coli* cells (Harris et al. 1994; Foster et al. 1996; Harris et al. 1996) and requiring DNA polymerase III (Foster et al. 1995; Harris et al. 1997) is also most easily accommodated by models in which RecBCD-mediated DSBR can prime replication (which leads to polymerase error and mutation (Harris et al. 1994; Rosenberg 1997; Lombardo and Rosenberg 1999). Here too, a direct demonstration of replicated recombinants has not yet been made. The generality of this mechanism is also uncertain because it is observed, so far, only in stressed and starving cells.

Third, the most suggestive previous evidence supporting a role for replication in DSBR is that the replication primosome assembly protein PriA is important for replication and is partially required for conjugational and transductional recombination. Its absence causes a roughly two-thirds reduction in recombination (Kogoma et al. 1996). This result is easily understood if replication is required for about 2/3 of RecBCD-mediated recombination, but this did not distinguish this hypothesis from the possibility that PriA, a DNA-binding protein, enhances recombination independently of its action in promoting replication. Although the biochemistry of PriA is consistent with a role in promoting replication during recombination (Liu et al. 1999), it is not yet known whether that is the role of PriA in recombination in vivo.

Other good arguments have been advanced (e.g. Smith 1991; Kuzminov 1995; Courcelle et al. 1997).

The mechanism of the replicational DSBR recombination in *E. coli*

Break-copy mechanisms for the replicational DSBR such as the one shown in Figure 2-5 are strongly supported by the results reported here. There is a close correspondence between the amount of newly synthesized DNA in the replicational recombinants with the distance from the crossover point to the end of the λ chromosome. This observation is compatible with and supportive of break-copy models in which the new strands segregate conservatively (Figure 2-5). However, alternatives are possible.

Alternative interpretations

First, in phage T4, one mode of replicational recombination, called "join-cut-copy", has been demonstrated (in addition to standard break-copy also done by T4) (Mosig 1998). The join-cut-copy events proceed only via leading strand synthesis. An invading 3' end synthesizes one new strand from the crossover point rightward (in diagrams such as in Figure 2-5) and then a T4-encoded endonuclease cuts the template molecule on the opposite strand at the crossover junction. The 3' end from this nick primes leading strand synthesis from the crossover point leftward (see Mosig 1998) This odd mechanism produces a recombinant that contains one new strand from the crossover point rightward and the other new strand from the crossover point leftward. As yet, no recombination nuclease is known to have this function in *E. coli* (but see Chiu et al. 1997) but we cannot rule this mechanism out. Further experiments will be required to distinguish break-copy from join-cut-copy modes of replicational recombination, and to address more directly models with conservative versus semiconservative segregation of strands.

Second, DNA replication pausing has been shown to lead to double-strand breakage in *E. coli* (Seigneur et al. 1998) in a process that requires Ruv proteins.

Could the role of replication in recombination reported here be in production of DSBs, which are necessary for RecBCD to load onto and recombine DNA? Three facts argue against this idea: first, such DSBs should not occur in cells lacking Ruv functions (Seigneur et al. 1998), whereas our requirement for replication in recombination is seen only in Ruv⁻ cells (Figures 2-4, 2-6). Second, in λ, the *cos* site is well documented to be the DSB site at which RecBCD loads, and to be required even when DNA replication is allowed (Kobayashi et al. 1982; Kobayashi et al. 1983; Kobayashi et al. 1984) (see Figure 2-5). Thus, it is most unlikely that the role of replication is to provide DSBs. Finally, this postulate does not predict the specific absence of break-join (central) recombinants among unreplicated molecules in *ruv recG* (Figure 2-6C), whereas break-copy models do.

Strand Polarity

Neither break-join, nor replicative mechanisms bear particularly on the polarity of RecA-mediated strand-invasion that creates bi-molecular strand-exchange intermediates (e.g. Figure 2-5B, C, D). The possibility that both 5' and 3' single-strand DNA ends created by RecBCD can invade was presented by Rosenberg and Hasting 1991, and supported by in vivo evidence of Hagemann and Rosenberg (1991), Miesel and Roth (1996), Razavy et al. (1996) and some biochemistry by Dutreix et al. (1991), Taylor and Smith (1995), Shan et al. (1997), and the hypothesis that only 3' ends can invade (as observed under different in vitro reaction conditions (e.g. Anderson and Kowalczykowski 1997) and in an unusual unimolecular reaction in vivo (Friedman-Ohana and Cohen 1998) can both be accommodated by our observation of roughly equal break-join and replicative recombination. For example, it has been hypothesized that 3' end-invasions might prime the replication in break-copy models whereas 5' end-invasions might lead only to break-join (Harris et al. 1996), in accordance with the rough equality (1:2) of 3' and 5' heteroduplex recombinants observed previously (Hagemann and Rosenberg 1991). These possibilities will require further study to address.

Replicational recombination in other organisms

The connection between recombination and replication is best established in bacteriophage T4, in which much of DNA replication requires homologous recombination functions (Dannenberg and Mosig 1981; Luder and Mosig 1982; Dannenberg and Mosig 1983; Formosa and Alberts 1986). Although no other system has yet provided as direct a demonstration of replicational recombination as the T4 system and the data for *E. coli* presented here, replicational recombination models are currently gaining favor in multiple systems in including in yeast (e.g. Strathern et al. 1995; Morrow et al. 1997; Bosco and Haber 1998; Holmes and Haber 1999) and mammalian cells (Harris et al. 1999) Such replicational DSBR could be an important source of nonreciprocal translocations, loss of heterozygosity, telomere extension, and other genome rearrangements important in formation of human cancers and aging (Ellis et al. 1995; Yu et al. 1996; Nugent et al. 1998; Haber 1999).

Why is either Ruv or RecG required for conjugational and transductional recombination?

In the phage λ assay system, replication can, in effect, substitute for the Ruv and RecG HJ processing systems of *E. coli*. However, this is not observed for the *E. coli* chromosome. Double mutants of any *ruv* gene with *recG* are recombination-deficient for *E. coli* conjugational and transductional recombination (Lloyd 1991) as if the replicational RecBCD-mediated mechanism cannot substitute in these processes (for views of the roles of Ruv and RecG in DSBR, see Lloyd 1991; Eggleston and West 1996; Harris et al. 1996). Several explanations are possible for this apparent discrepancy. First, it is possible that conjugation and transduction are strictly non-replicational events. Second, it is also possible that for some reason, DNA replication forks assembled at recombination junctions are less processive than those that start at a replication origin (Bosco and Haber 1998), such that the 48 kb λ genome can be replicated by recombination but the 4.5 mb *E. coli* genome cannot. A more unifying class of explanation than either of these is presented in Figure 2-7.

The replication forks initiated at recombination intermediates should be different from those that start at a replication origin in that they are associated with a Holliday junction behind the advancing fork (Figure 2-7). The migration of Holliday junction-containing replication bubbles around the *E. coli* chromosome might require branch migration proteins such as RecG or RuvAB (Figure 2-7B, C). λ might escape this need either because the distance is shorter, or because some other activity substitutes for Ruv/RecG-mediated branch migration of the replication bubble. For example phage DNA packaging occurs concurrently with RecBCD-mediated recombination of the λ chromosome (see Figure 2-7A) because the DSB made to initiate packaging is the same one for RecBCD-loading (Kobayashi et al. 1984; Myers and Stahl 1994). The packaging apparatus travels in the same direction (rightward Figure 2-7A) as the branch migration that would be necessary to move the junction rightward. Perhaps the packaging apparatus can move the junctions at the forks for λ. Alternatively, because a replication bubble will not encounter any replication terminus in λ DNA (as it would in the *E. coli* chromosome), replication forks started at a recombination intermediate could proceed around the entire l chromosome (the circle in Figure 7A) and the replisome then might push the junction rightward

(Morgan and Severini 1990). For λ, the junction need only move past the next packaging origin encountered (*cos*, Figure 2-7A) to produce a packagable replicated recombinant. Although, other explanations are also possible, this one and variations on the theme in Figure 2-7 (see Bosco and Haber 1998) are simple in that they do not require any special properties of the replication associated with recombination that are not seen for replication in general. These models also make testable predictions. Further work will be required to address the possibilities raised by findings reported here.

Materials and Methods

Bacterial and phage strains. All bacterial strains used are *E. coli* K12 derivatives and are listed in Table 2-3. New genotypes were constructed using standard phage P1-mediated transduction (Miller 1992). The presence of *recA, recG, ruvA, ruvB,* and *ruvC* alleles was confirmed by the increased ultraviolet light (UV) sensitivity phenotypes conferred by these mutations. For all *ruv recG* double mutants, the presence of both alleles was confirmed by verifying the extreme UV sensitivity characteristic of strains lacking both Holliday junction processing systems (Lloyd 1991). The *ruvC recG* double mutant SMR650 was constructed from SMR632 as follows. First, *ruvC53 eda-51*::Tn*10* (Lloyd 1991) was introduced by transduction with phage P1 grown on CS85. Second, *recG258::*Tn*10*minikan was introduced by transduction with P1 grown on strain RDK2655 (Lloyd and Buckman 1991, obtained from R. Kolodner). SMR3124 was constructed similarly except with a different *ruv*. The P1 donor for SMR3124 was RDK2641 carrying *ruvA59*::Tn*10* (Shurvinton et al. 1984). SMR3669, a *ruvC recG* strain also lacking *rusA*, was constructed by introducing Δ*rusA*::*kan* from strain AM821 (Mahdi et al. 1996) *ruvC53 eda-57*::Tn*10*::*cam* (obtained from a transductant of CS85 x P1

RM5258), and *recG162 zib-636*::Tn*10* (Storm et al. 1971) in that order into SMR632. The presence of Δ*rusA*::*kan* was confirmed by PCR as described (Mahdi et al. 1996). The UV sensitivity of the *ruvC recG rusA* strain was similar to that of a *ruv recG* strain.

Strain SMR4292 lacking the *rac* prophage was constructed by transducing the *rac*⁻ Su⁻ *rec*⁺ strain JC11450 with *ruvC53 eda-51*::Tn*10* (Lloyd and Buckman 1991) and *recG258*::Tn*10minikan* (Lloyd and Buckman 1991). Strain SMR4594 carrying the temperature-sensitive *dnaE* allele *dnaEts486* was constructed by transducing SMR632 with P1 grown on SMR540 (lab collection, allele from R. Maurer, Case Western Reserve University). A *ruvC recG dnaEts* strain was made by transducing SMR4594 with *ruvC53 eda-51*::Tn*10* (Lloyd and Buckman 1991) and *recG258*::Tn*10 minikan* (Lloyd and Buckman 1991) to make SMR4600. The *recA* derivative of SM4594 was made by introducing the Δ*(srlR-recA)*::Tn*10* allele by transduction with P1 grown on SMR624 (Harris et al. 1994).

A set of *ruvC recG* strains in which λ replication could be blocked was created by first lysogenizing SMR632 with λ*Jts15 red3 gam210* Δ*nin5 Sam7* [λSR459 (Sawitzke and Stahl 1997)], followed by transduction to kanamycin resistance with P1 grown on a *grpD55 malF*::Tn*10*::*kan* (Bull and Hayes 1996) strain to make SMR3731. *grpD55* is an allele of *dnaB* that blocks λ replication by lack of interaction with λ O and P replication proteins, but has no effect on *E. coli* replication (Bull and Hayes 1996). Lysogeny was confirmed in the *grpD55* strain by demonstrating immunity to a λ *imm21*-phage P22 hybrid that carries the *18* and *22* genes of P22 (equivalents of the λ *O* and *P* genes). A *ruvC recG* derivative of SMR3731 was made by transducing *ruvC53 eda57*::Tn*10*::*cam* (obtained from a transductant of CS85 x P1 RM5258) and *recG162 zib-636*::Tn*10* (Storm et al. 1971).

λ phages are either from the λSR collection or were gifts from F.W. Stahl or S. Hayes. λ *cI857* (*18, 22*) P22 (Bull and Hayes 1996) was used as the killer phage for our screen for lysogens in *grpD55* mutant cells at 42°. Phage genotypes used in crosses to measure the frequency of recombinants (Figures 2-2 and 2-3, Table 2-2) are λ *Δb527 red3 gam210 cI857 Chi*$^+$ C *Δnin5 Sam7* (Chi$^+$ *nin*$^-$); λ *Δb527 red3 gam210 cI857 cII68 Δnin5 Sam7* (Chi° *nin*$^-$); λ *bio1 Δnin5* (Chi$^+$ and/o *nin*$^-$) from (Razavy et al. 1996), and λ *Δb2 red3 gam210 cI857 Sam7* (*nin*$^+$); λ *bio1* (*nin*$^+$). The phage genotype used for experiments in Figure 2-4 was λSR27, *bio1 Δnin5*. The phages used in Figure 2-6 are MMS1816, λ *Jts15 int4 red3 gam210 cI857 Δnin5*; MMS1817, λ *int4 red3 gam210 Δnin5 Sam7*, and they recombine in the presence of homoimmune prophage MMS2076, λ *Jts15 red3 gam210 Δnin5 Sam7*, with helper packaging functions provided by MMS2084, λ *Jts15 int4 red3 gam210 imm*^{434} *Δnin5 Sam7* (Sawitzke and Stahl 1997).

Growth of phage stocks and *E. coli* cultures

dnaEts strains were grown at 28°. *ruv recG* double mutants are slow growing and form small colonies, such that cultures are prone to accumulation of faster growing and larger mutant colonies carrying suppressor mutations as well as true reversions (Lloyd and Buckman 1991; Harris et al. 1996). *ruv recG* double mutant strains were grown at 32° to avoid the accumulation of suppressors normally associated with growing these strains at higher temperatures (Harris et al. 1996). The UV and drug sensitivity phenotypes of all strains were confirmed for cultures used in each experiment (and/or for approximately 30 colonies from a given culture). Cultures were also routinely monitored for possible accumulation of suppressors or revertants as described previously (Harris et al. 1996).

λ phage stocks (carrying light isotopes) were grown, and plaque assays performed according to standard procedures (Murray 1983). Stocks of λ phage density labeled with ^{13}C and ^{15}N were grown according to procedures of Stahl et al. (1972a) on prototrophic bacteria, for 12-14 hrs at 32°.

Determination of recombination frequency

λ crosses to quantify the frequency of recombination were performed as described previously (Razavy et al. 1996), except that log phase cultures in which mixed infections were carried out were grown at 32° and 10-30 µl of the frozen bacterial cultures in our collection were used to inoculate the broth for growing each culture to log phase. A cesium formate density gradient for each cross lysate was then prepared, centrifuged to equilibrium, and collected as two drop fractions into 1 ml TB each. The titers of phage in the fractions collected were determined by plating the appropriate dilutions of each fraction on the $SuIII^+$ strain KR3a for total phage, and on the $SuII^+$ strain AFT196 for λS^+ recombinants.

λ recombination assay in the absence of DNA replication

Density-labeled (^{13}C, ^{15}N) λ *red gam nin* (λSR27) were infected into *E. coli* strains carrying the *dnaEts* allele at the non-permissive temperature of 43.5°. Cells were first grown at 28° to 2×10^8 cell/ml by inoculating 10-30 µl of the frozen stock into 10 ml of TB with 1% yeast extract, 0.2% maltose, and 0.01 mg/ml vitamin B1. Additionally, 25 µg/ml kanamycin was added to this broth for the growth of *ruv recG* strains (to avoid accumulation of *recG* revertants formed by transposon excision). Typically, 10-13 hours was required for cells to reach the correct density. Cell counts of each culture were measured using Petrauff Hausser counting chambers. Cells and the phage mixes were then pre-incubated at 43.5°

for 15 minutes prior to infections. Cells were infected with density-labeled λ at a multiplicity of 10 phage per cell. The phage and cell mixtures were bubbled vigorously for 30 minutes, then diluted with 4 ml of prewarmed TB with additions (above), and the mixtures were bubbled for another 35 minutes at 43.5°. The cell-phage mixtures were then diluted by adding 5 ml of cooled (4 degrees) TM and transferred to precooled centrifuge tubes and pelleted. Pellets were resuspended in 2 ml of chilled broth (as above). Lysozyme and chloroform were added to lyse cells and release the phages. Cell debris was pelleted and the supernatants collected.

A density gradient was prepared for each lysate (McMilin and Russo 1972). Two drop fractions were collected into 1 ml TB and phage titers were determined for each fraction on SMR423.

Assay for central and end λ recombinants formed under conditions of partial replication-block.

Partial replication block was achieved by homoimmune repression and heteroimmune helper phage infection, as described (Sawitzke and Stahl 1997), except that our *E. coli* strains also carried the *grpD55* mutation. *grpD55* encodes a DnaB helicase that does not interact with the λ replication proteins (Bull and Hayes 1996). In the absence of this allele, λ replication could not be blocked sufficiently in *ruv recG* lysogens to allow resolution of any unreplicated phage (HH peaks). Also, the cells were grown slowly at 32° to avoid the accumulation of suppressor mutants. Recombinants were assayed on strains JAS36 and JAS38 as described (Sawitzke and Stahl 1997).

Table 2-1. Efficiency of plating of λ *red gam* on *ruv recG* deficient *E. coli* strains

E. coli strains	eop ± SD[c]		
	λ Chi^{+} [d]	λ Chi^{o} [e]	λ *nin*$^{-}$ [f]
rec^{+}[a]	1.0	1.0	1.0
ruvA recG[a]	1.1 ± 0.3	0.98 ± 0.2	0.96 ± 0.1
ruvC recG[a]	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
rec^{+}[b]	1.0	1.0	1.0
ruvA recG[b]	0.91 ± 0.3	0.88 ± 0.2	0.91 ± 0.1
ruvC recG[b]	0.95 ± 0.1	0.87 ± 0.5	0.86 ± 0.1

[a] Isogenic derivatives of strain FC40 published previously (Harris et al. 1996)(Table 2).

[b] Isogenic derivatives of strain SMR632 (Materials and methods, Table 2).

[c] Efficiency of plating (eop) for each strain was determined by dividing the λ titer on the *ruv recG* strain by its titer on the *rec*$^{+}$ strain. This number was then corrected for the viability of the cultures of cells on which the plaques were assayed by dividing by the relative viability of the strain. The relative viability of each strain was determined as follows: (viable cell count of *ruv recG*/total cell count of *ruv recG*) / (viable cell count of *rec*$^{+}$ /total cell count of *rec*$^{+}$). Each determination is a mean (± standard deviation) of 3 independent experiments in which hundreds of plaques were counted. The absolute viability of the *rec*$^{+}$ strains were [a]0.9 ± 0.04, and [b]0.7 ± 0.1 cfu/cell counted (mean ± SE for the 3 experiments reported) and the relative viabilities were [a]0.4 ± 0.1, and [b]0.4 ± 0.1 for their *ruvA recG* derivatives , and [a]0.4 ± 0.1, and [b]0.4 ± 0.7 for their *ruvC recG* derivatives. These values are as reported (Lloyd 1991).

[d] λ Δ*b1453 cI857 Chi*$^{+}$*C* (The Δ*b1453* deletion removes *int*, *red*, and *gam*.)

[e] λ *Δb1453 cI857 cII68*

[f] λ *bio1 Δnin5* (The *bio1* substitution removes *int*, *red*, and *gam*. *Δnin5* removes λ analogs of *E. coli* recombination genes, discussed in the text.)

Table 2-2. Recombination of λ *red gam* Δ*nin* in *rac*$^-$ or Δ*rusA* *E. coli* strains

Strain[a]	Expt	Percent[c] homologous recombination (mean ± SD)	Strain[b]	Expt	Percent[c] homologous recombination (mean ± SD)
rec$^+$	1	5.2 ± 0.6	*rac*$^-$ *rec*$^+$	1	4.0 ± 0.5
	2	6.3 ± 0.8		2	4.7 ± 0.4
ruvC recG Δ*rusA*	1	2.9 ± 0.3	*rac*$^-$ *ruvC recG*	1	2.6 ± 0.3
	2	3.2 ± 0.8		2	2.3 ± 0.7

[a] These strains are SMR 632 and SMR 3669 (Table 2-3).

[b] These strains are JC11450 and SMR 4292 (Table 2-3).

[c] Percentages of homologous recombination are calculated as described in Figure 2-2 using Chi^o Δ*nin5* phage (Figure 2-2). Recombination is measured in the λ *gam* to *S* interval.

Table 2-3. Bacterial strains

Strain	Relevant properties	Source or reference
JC11450	*rac*$^-$ *rec*$^+$	A. J. Clark, Berkeley
594	Su$^-$ *rec*$^+$	(Weigle 1966)
C600	SuII *rec*$^+$	(Appleyard 1954)
AFT196	C600 *Δ(srlR-recA)306::*Tn*10*	Lab collection
KR3a	SuIII *recA*	Lab collection
RDK2641	*ruvA59::*Tn*10*	R. Kolodner
CS85	*ruvC53 eda-51::*Tn10	R.G. Lloyd, *via* R. Kolodner
RDK2655	*recG258::*Tn*10minikan*	R. Kolodner
RM5258	*eda-57::*Tn*10::cam*	(Foster et al. 1996)
SMR423	C600 SuII III *recD1903::Tet hsdrK*$^-$*mK*$^+$	Lab collection
SMR632	594 *hsdrk*$^-$ *mk*$^+$	Lab collection
SMR650	SMR632 *ruvC53 eda-51*::Tn*10 recG258*::Tn*10 minikan*	This work
SMR3124	SMR632 *ruvA59*::Tn*10 recG258*::Tn*10minikan*	This work
AM821	*ΔrusA::kan*	(Mahdi et al. 1996)
SMR3669	SMR632 *ruvC53 eda-57*::Tn*10::cam recG162 zib-636*::Tn*10 ΔrusA::kan*	This work
SMR4292	*rac*$^-$ *ruvC53 eda-51*::Tn10 *recG258*::Tn*10minikan*	This work
SMR4594	SMR632 *dnaEts486 zae*::Tn*10d-Cam*	This work
SMR4600	SMR632 *dnaEts486 zae*::Tn*10d-Cam ruvC53 eda-51*::Tn*10 recG258*::Tn*10minikan*	This work
SMR4601	SMR632 *dnaEts486 zae*::Tn*10d-Cam Δ(srlR-recA)306*::Tn*10*	This work
SMR3731	SMR632 *grpD55 malF*::Tn*10::kan* (λ *Jts15 red3 gam210 Δnin5 Sam7*)	This work
SMR3732	SMR632 *grpD55 malF*::Tn*10::kan recG162 zib636*::Tn*10 ruvC53 eda57*::Tn*10::cam* (λ *Jts15 red3 gam210 Δnin5 Sam7)*	This work

JAS36	C600 (λ*Jts15 red3 gam210 imm434 Δnin5 Sam7*)	(Sawitzke and Stahl 1997)
JAS38	*Δ(srlR-recA)*306::Tn*10 recD1009* (λ *Jts15 red3 gam210 imm434 Δnin5 Sam7*)	(Sawitzke and Stahl 1997)
FC40	*ara* Δ(*lac-pro*)XIII *thi* Rif^R [F' *lacI33 WlacZ proAB*]	(Cairns and Foster 1991)
RSH45	FC40 *ruvC53 eda51::*Tn*10 recG258*::Tn*10minikan*	(Harris et al. 1996)
RSH160	FC40 *ruvA59::*Tn*10 recG258::*Tn*10minikan*	(Harris et al. 1996)

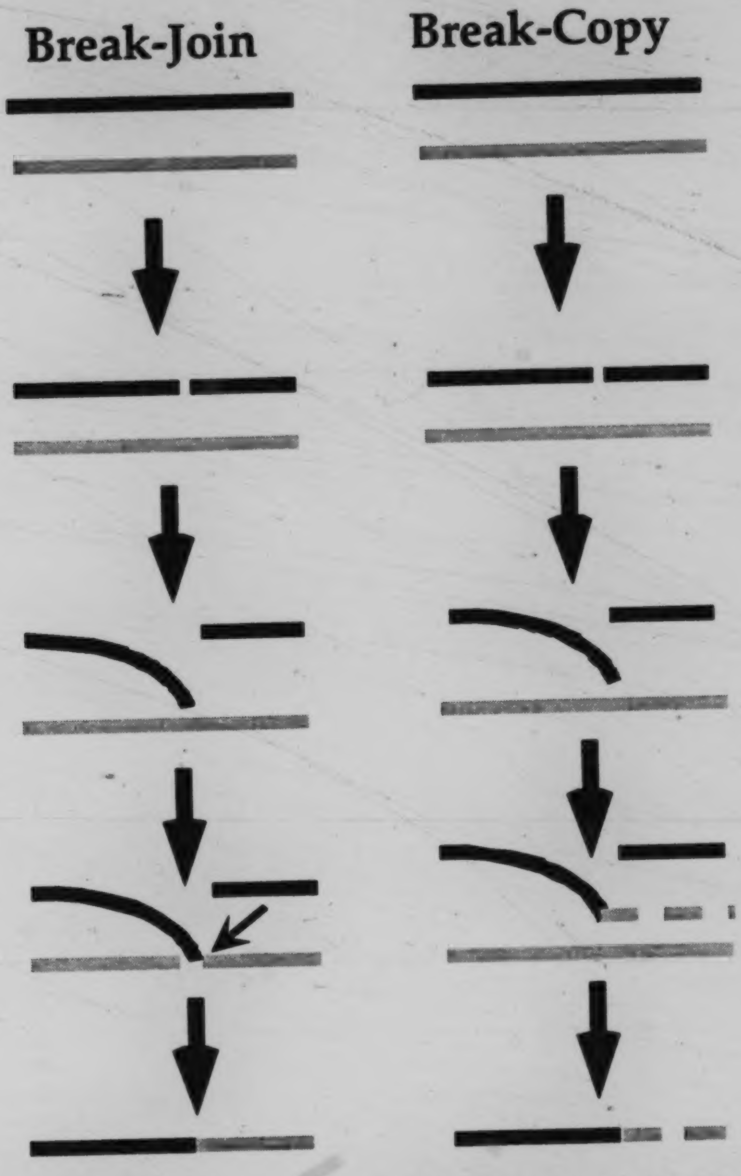


Figure 2-1. Two early general models for homologous recombination (adapted from Meselson and Weigle, 1961). Each line represents duplex DNA. Dashed lines represent newly synthesized DNA. Solid lines represent "old" parental DNA. HJ processing (thin arrow) indicates action of HJ resolution proteins, including an endonucleolytic cleavage (such as RuvC performs) to break the invaded molecule (gray) and allow its ligation to the black fragment. No strand polarities are shown because specific polarities are not implied by either model (see Discussion).

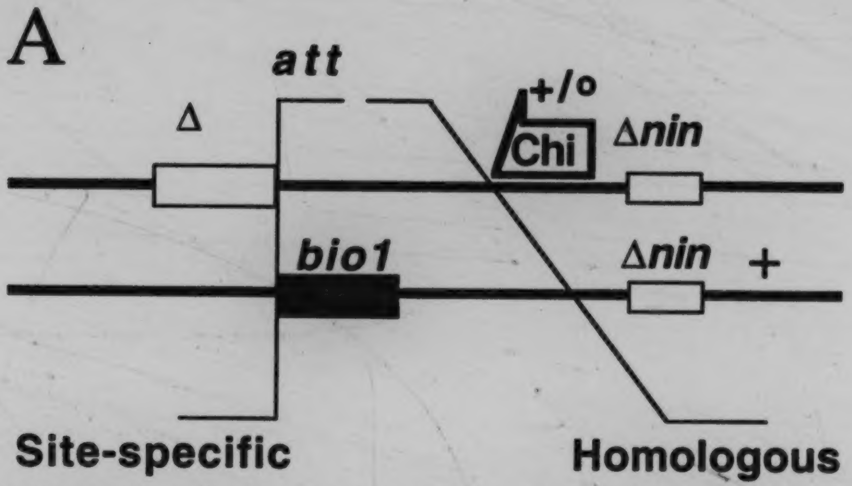
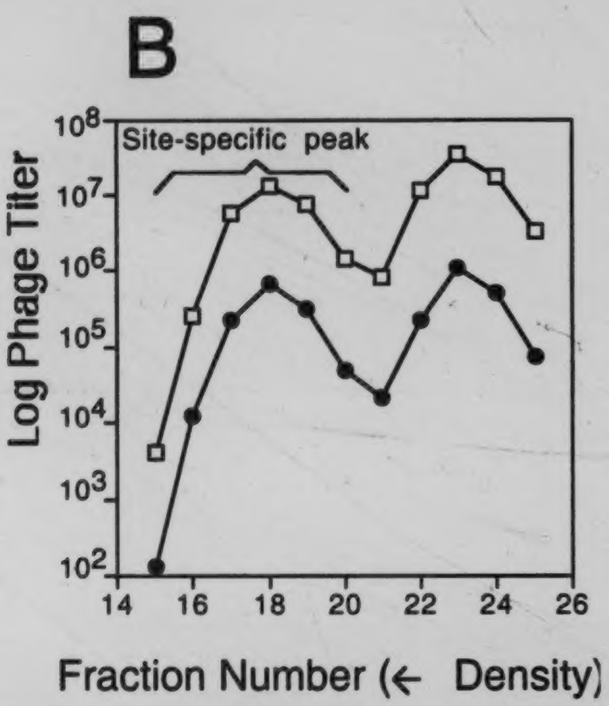


Figure 2-2. Design of λ crosses used to measure the frequency of recombination in rec^+ and *ruv recG* cells. (*A*) The strategy for this assay is described in the text. Strains and methods used are those of Razavy et al.(1996). This general diagram shows all of the relevant genetic markers used. The open box represents either of two different deletions (Δ*b527* or Δ*b2*, Materials and methods) both starting from the core *att* site and removing DNA to its left. The filled box represents a deletion/substitution (*bio1*) starting from the core *att* site and removing DNA to its right, resulting in a net loss of approximately 2 kb of DNA. The arrow indicates the direction of the Chi sequence; "+" indicates the wild-type copy of the *S* gene; the other parent carries *Sam7*. Crosses performed (Figure 2-3) varied the presence/absence of the Chi site, Chi^+C, and of the *nin* deletion, Δ*nin5*. All phage are *red gam*, the top phages by carrying *red3 gam210* mutant alleles, and the bottom phages by virtue of the *bio1* substitution (see Materials and methods and Razavy et al. 1996 for full genotypes).



(*B*) A representative cesium formate equilibrium density gradient of a cross progeny showing the denser peak formed by site-specific recombination, which contains neither Δ nor *bio1* net deletions (fractions 15-20). The next lighter peak (fractions 21-25) includes the top parental phage (*A*) plus its S^+ recombinant derivatives. Open squares and filled circles represent total phage (*Sam7* and S^+) and S^+ recombinants, respectively. These plaques were assayed on SuIII$^+$ *recA* (for "total" phage) and SuII$^+$ *recA* (for S^+ recombinants) cells, which do not allow plaque formation of phage with the *bio* substitution but do allow the *gam* (amber)*210* carriers to form plaques (Materials and methods). Thus, we do not see the double-deletion (Δ*bio1*) site specific recombinant peak. To calculate the frequency of λ S^+ homologous recombinants among site-specific recombinants, the titer of λ S^+ in each fraction (15-20) is divided by the total titer in that fraction, and the mean ± SD for all the fractions in the peak is expressed as a percentage (Figure 2-3 and Table 2-2). The data shown are from a cross in *rec*$^+$ SMR632 cells using Chi° *nin*$^+$ phage (Materials and methods).

Figure 2-3. RecBCD-pathway recombination of λ in the absence of RuvC and RecG. The three bar graphs summarize the results of three different experimental designs, measuring the efficiency of λ recombination in *rec*$^+$ and *ruvC recG* cells. For each design, we used a different set of phages and the relevant λ genotype (*nin*$^+$, Δ*nin*, and Δ*nin* Chi$^{+/o}$) is shown above each bar graph. The significance of these genotypes is described in the text. Each bar represents the mean percentage of homologous recombination among site-specific Int-mediated recombinants (± SD; calculated as described in Fig. 2). Three independent experiments were performed for *nin*$^+$ and Δ*nin* crosses. Two experiments were performed for the Δ*nin* Chi$^{+/o}$ cross. The deletion Δ*nin* shortens the DNA segment whose recombination is assayed (see Fig. 2), and therefore necessarily decreases the percent recombination relative to *nin*$^+$ crosses. Thus, the important comparison for both *nin*$^+$ and Δ*nin* crosses is between presence or absence RuvC RecG in each.

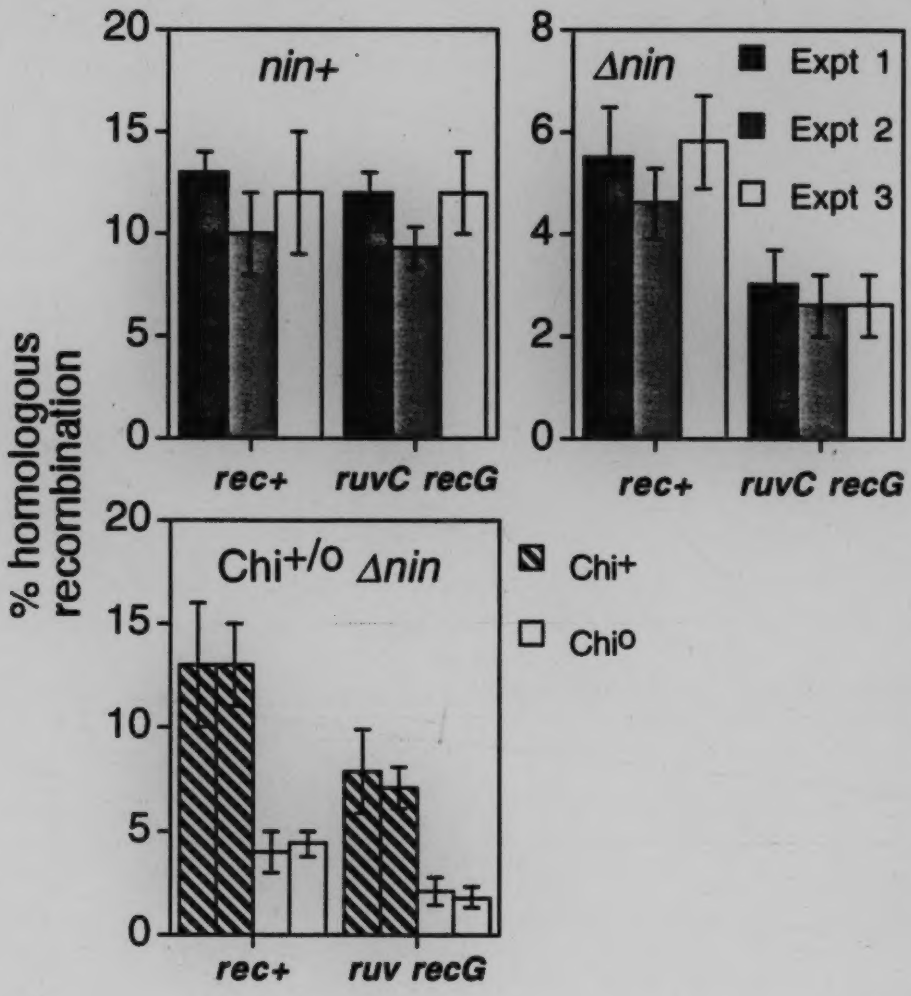
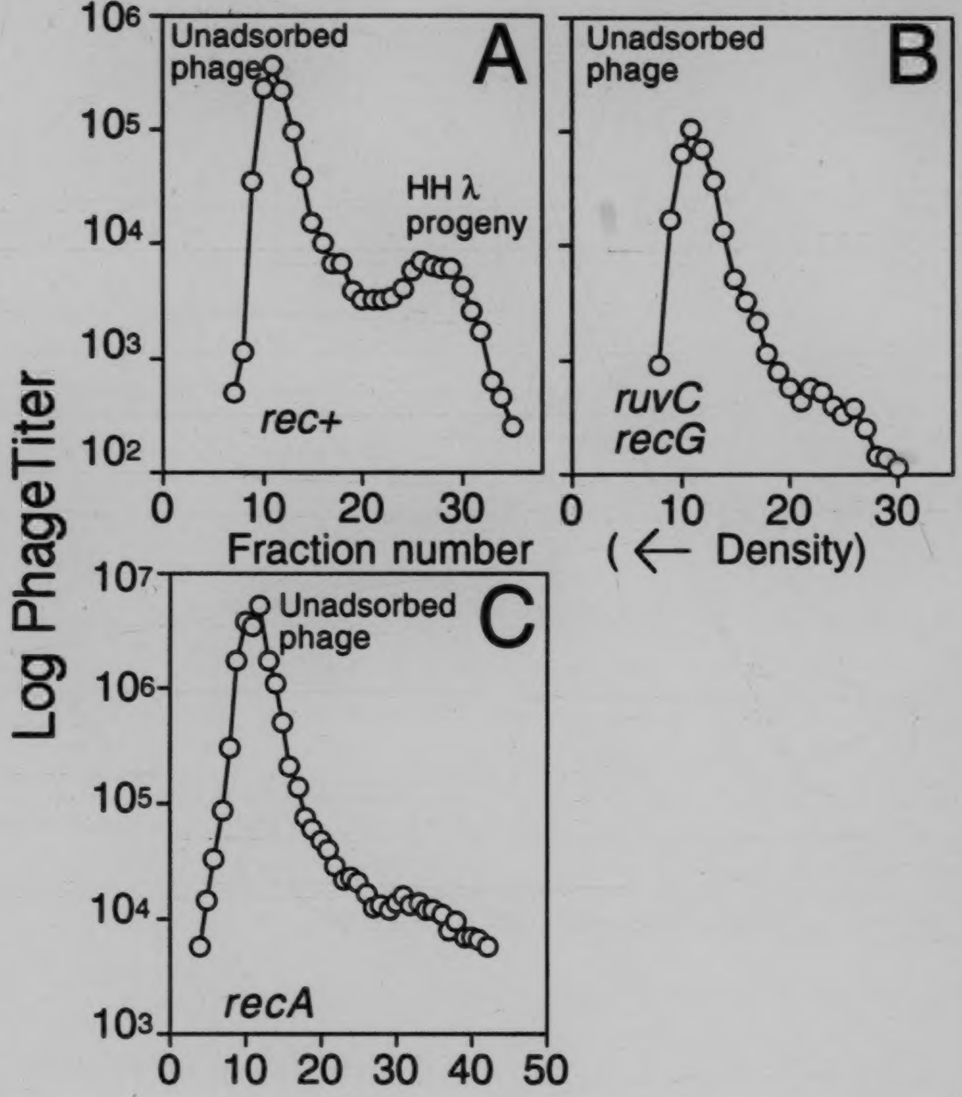

% homologous recombination
20
15
10
5
0
nin+
rec+
ruvC recG
8
6
4
2
0
Δnin
Expt 1
Expt 2
Expt 3
rec+
ruvC recG
20
15
10
5
0
Chi+/o Δnin
Chi+
Chio
rec+
ruv recG

Figure 2-4. λ Progeny formation in the absence of DNA replication requires RuvC/RecG. λ progeny formation was used to assay recombination. Replication was blocked by infecting cells that carry a temperature sensitive allele of the *dnaE* gene with density-labeled λ (λSR27) at 43.5°C, at which temperature we obtain a complete replication-block. These graphs represent the titers of plaque forming λ in the fractions of a density gradient obtained following each infection. The densest fractions are to the left on each graph. The first peak in all gradients contains unadsorbed λ. These phage carry heavy protein coats and heavy (HH) DNA. They did not enter the light cells and therefore, are not part of the λ progeny. (*A*) Density gradient of infection in *rec*$^+$ cells. Two peaks are apparent. The second peak contains λ progeny which have entered the cell, recombined and packaged. These carry light protein coats and heavy (HH) DNA. No other peaks are detected because of the replication block. (*B*) *ruvC recG* cells. Few or no λ progeny are detected. (C) *recA* cells. Few or no λ progeny are detected.


Log PhageTiter
A
Unadsorbed phage
HH λ progeny
rec+
10^6
10^5
10^4
10^3
10^2
0
10
20
30
Fraction number
B
Unadsorbed phage
ruvC
recG
0
10
20
30
(← Density)
C
Unadsorbed phage
recA
10^7
10^6
10^5
10^4
10^3
0
10
20
30
40
50

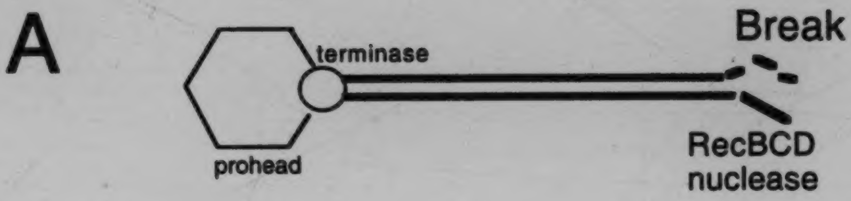
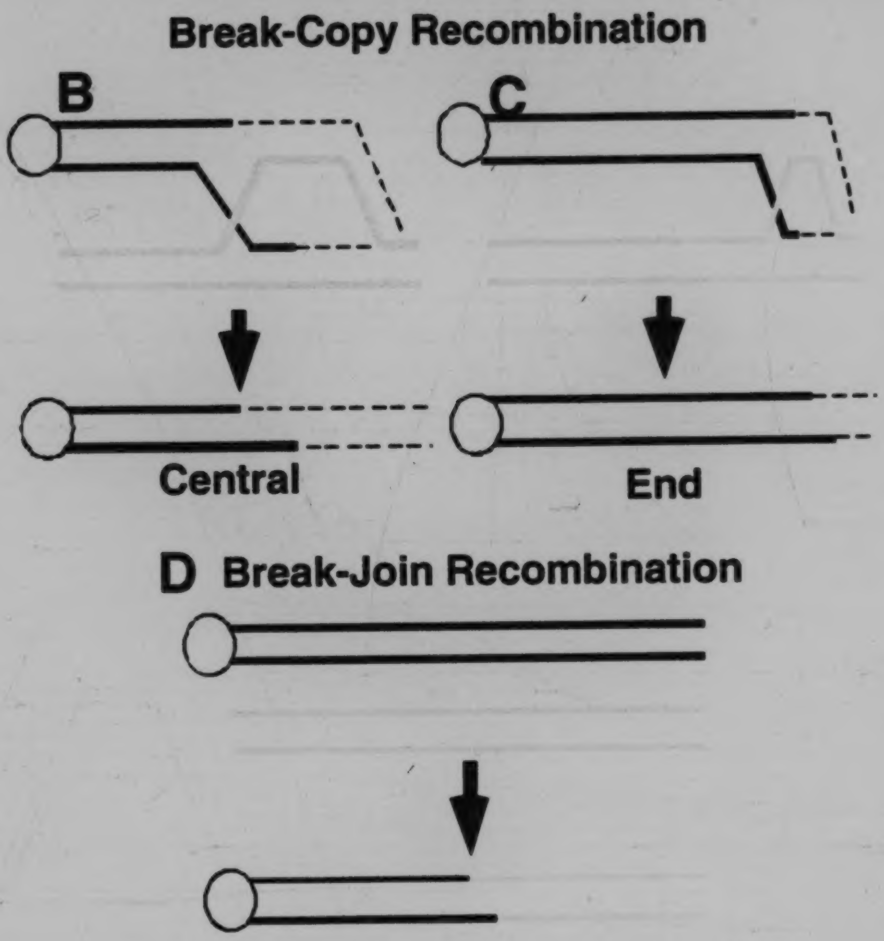


Figure 2-5. Predictions of break-copy and break-join recombination models. The thick lines represent parental DNA (black and gray). The dashed-lines represent newly synthesized DNA. Specific strand-polarities are not indicated because no specificities are implied by either model depicted (see Discussion). (*A*) The phage λ DNA molecule is linearized during DNA packaging by the endonuclease terminase (white circle) which remains bound to the λ left end after DNA cleavage (Kobayashi et al. 1982; Kobayashi et al. 1983). (Hexagon represents the phage prohead attached to terminase during packaging and concurrent DSBR recombination (Kobayashi et al. 1984).) Only the right end is available for DSBR (Kobayashi et al. 1982; Kobayashi et al. 1983) which begins with degradation leftward by RecBCD exonuclease, reviewed by (Kowalczykowski et al. 1994; Myers and Stahl 1994). (Note that Chi sites [not shown] are recombination hotspots in this pathway because when RecBCD reaches Chi, Chi decreases RecBCD nuclease activity allowing the DNA there to recombine (reviewed by Myers and Stahl 1994; Rosenberg and Motamedi 1999).



(B,C,D) In a break-copy process, the degraded right end initiates a replication fork(B, C). Semi-conservative replication of density labeled DNA to the end of the chromosome followed by the conservative segregation of the new strands (shown) would produce recombinant molecules with the following densities: (*C*) End recombinants inherit mostly parental DNA (and would be expected to band in or near the heavy-heavy [HH] peak in a density transfer experiment (see Fig. 6*B* and *C*). (*B*) Central recombinants would contain roughly half parental and half newly synthesized DNA (and would band in the heavy-light [HL] peak in a density transfer experiment (see Fig. 6*B* and *C*). (*D*) In break-join, recombination intermediates are resolved by the Holliday junction resolution systems. The recombinant molecules inherit only atoms from parental DNA; no new synthesis is required to complete the recombination reaction. These central recombinants would fall into the first few fractions of the "HH" peak in a density transfer experiment (see Fig. 6*B*).

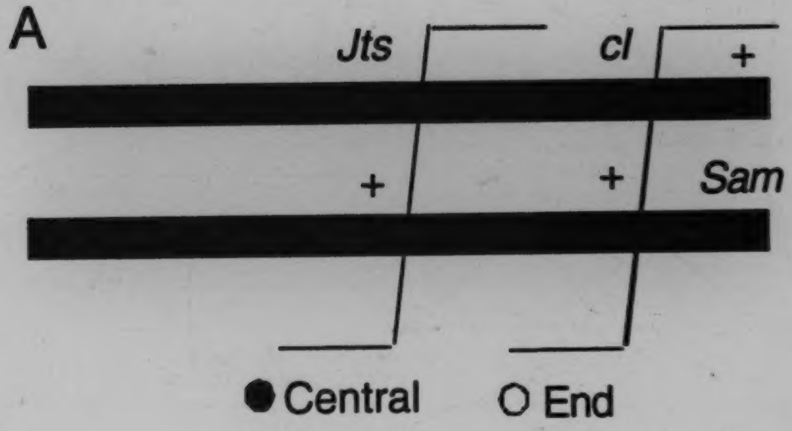
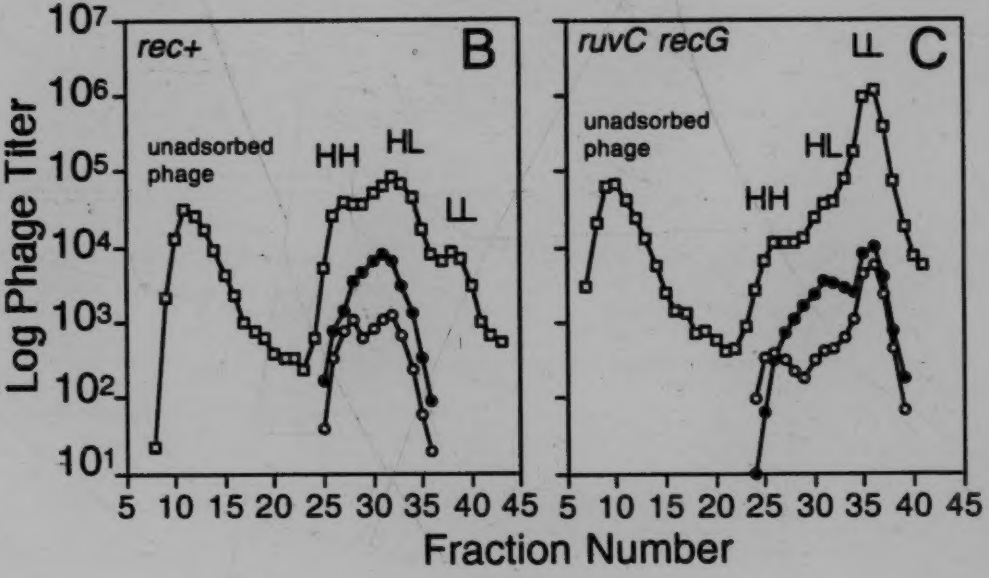


Figure 2-6. Extent of DNA replication in central and right-end λ recombinants in crosses with some replication allowed in *rec*$^+$ and *ruv recG* cells. These crosses were conducted under partial replication-block (Materials and Methods) to allow visualization of any break-copy recombinants. If full replication-block is used, no HH peak is visible for *ruv recG* (Fig. 4*B*). (*A*) The relevant genotypes of phages used in this experiment. These phage (Sawitzke and Stahl 1997, see Materials and Methods) carry the *nin5* deletion and are marked to allow selection of *J*$^+$ *S*$^+$ recombinants from which central (*J*$^+$ *cI* *S*$^+$, clear, filled circle) and right end (*J*$^+$ *cI*$^+$ *S*$^+$, turbid, open circle) recombinants are enumerated.



(*B, C*) Density labeled (^{13}C, ^{15}N) phage were allowed to recombine under partial replication block (Materials and methods) and the progeny centrifuged to equilibrium in cesium formate density gradients, which were fractionated. Note that the progenies band into unreplicated, heavy-heavy (HH), and replicated, heavy-light and light-light (HL and LL) peaks. Total λ (open squares), and J^+ S^+ recombinants were assayed (Materials and methods), and central (filled circle) and right end (open circle) recombinants counted. The first peak (leftward) in these experiments represents unadsorbed phage (heavy coats and HH DNA) which are not part of the λ progeny. (*B*) Density gradient of the λ cross in *rec*$^+$ cells. Discussed in the text. (*C*) Density gradient of the λ cross in *ruvC recG* cells. Discussed in the text.

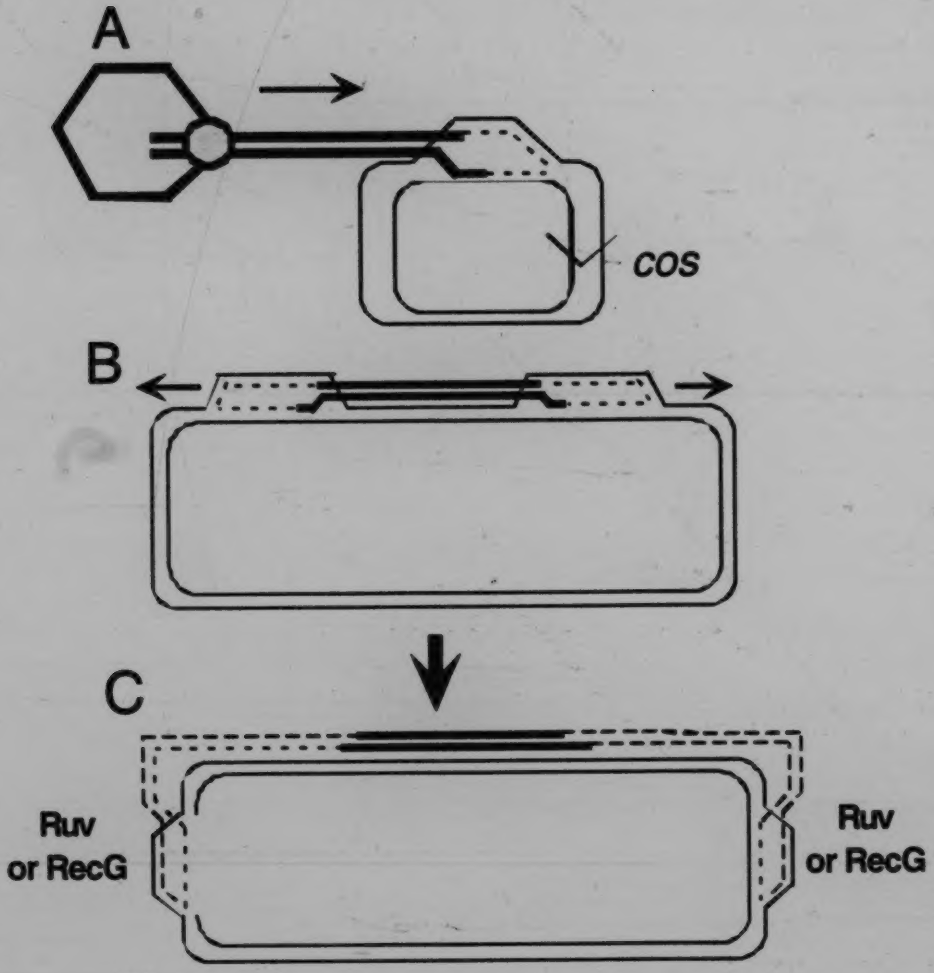


Figure 2-7. Break-copy models illustrated for RecBCD-mediated recombination of λ (*A*) and *E. coli* (*B, C*) genomes. Model discussed in the text. The hexagon represents the prohead during λ DNA packaging from *cos* to *cos*. The ball represents the terminase protein which linearizes λ, binds the prohead, and packages the DNA.

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Chapter 3[*]

Evidence for the occurrence of two distinct RecBCD-mediated recombination intermediates in vivo: One requires processing via RuvABC, whereas the other is processed independently of an endonucleolytic cleavage.

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Introduction

The faithful passage of genetic information from parent to offspring is crucial for the fitness and survival of any species. It requires the error-free duplication of the parental genome, by DNA replication, followed by the transmission of a new copy to the offspring. Any impediment to this process undermines the survival of the species.

DNA double-strand breaks (DSBs) occur naturally and frequently in cells (Skalka 1974; Kuzminov 1995; Seigneur et al. 1998; Michel 2000). They impede DNA replication , and act as entry sites for various DNA degrading enzymes (such as exonucleases) to the chromosome (Michel et al. 1997). The accumulation of these lesions threatens the integrity of the genome, and ultimately causes cell death.

Obviously, mechanisms have evolved to repair these lesions. The most conservative method for their repair is via recombination-dependent DNA double-strand break-repair (DSBR) (reviewed in Clark and Sandler 1994; Kanaar and Hoeijmakers 1998; Haber 1999). This mechanism is conservative because repair is mediated through the exchange of information between homologous molecules, with no net loss of genetic material. The conservative nature of DSBR is unique because, for example, in another repair pathway, non-homologous end joining, the DSB is repaired, independently of homologous recombination proteins, by being physically connected to any available DNA end (Lobrich et al. 1995). This often results in gross chromosomal translocations, and loss of genetic information at the break site (reviewed in Tsukamoto and Ikeda 1998; Haber 1999).

In the bacterium *Escherichia coli*, the dominant route for repairing DSBs is via RecBCD-mediated DSBR (Kowalczykowski et al. 1994; Myers and Stahl 1994;

Kowalczykowski 2000). Most of the enzymes that work in this pathway have been identified (reviewed in Lloyd and Low 1996; Rosenberg and Motamedi 1999; Kowalczykowski 2000). RecA and RecBCD proteins operate to align (synapse) homologous molecules, forming bi-molecular recombination intermediates, such as Holliday junctions (HJ). In *E. coli,* two apparently independent pathways operate to process DNA intermediates into mature recombinant DNA: the RuvABC and the RecG systems (Lloyd 1991). The RuvAB complex (made of RuvA and RuvB proteins) and RecG protein specifically bind and branch migrate HJs made by RecA (Parsons et al. 1992; Tsaneva et al. 1992; Whitby et al. 1993). RuvC is an endonuclease, exclusively cutting DNA at four-way junctions, producing recombinant molecules (Connolly et al. 1991; Iwasaki et al. 1991; Bennett et al. 1993). RuvC has been shown to work in a complex with RuvA and RuvB proteins (van Gool et al. 1998), forming the RuvABC resolvasome in vitro (van Gool et al. 1999). The catalytic activities of the RuvABC complex are sufficient to branch migrate and resolve recombination intermediates into mature recombinant DNA products (reviewed in West 1994; Kuzminov 1996). RecG is thought to act in an independent mechanism for processing recombination intermediates in *E. coli* (Lloyd 1991). Its branch migration activity is thought to be sufficient to process recombination intermediates in vitro (Lloyd and Sharples 1993; Whitby et al. 1993).

Historically, recombination-dependent DSBR in *E. coli* was thought to proceed by the breaking and re-joining of DNA molecules (break-join) exclusively (Meselson and Weigle 1961; Meselson 1964; McMilin and Russo 1972; McMilin et al. 1974), with no requirement for DNA synthesis. The discovery of HJ-specific proteins, specifically RuvC, supported break-join DSBR models by providing the enzymatic activity predicted for the resolution of HJ intermediates (e.g. Thaler and Stahl 1988; Lloyd and Low 1996). However, recent evidence suggested the existence of an alternate recombination-dependent repair pathway, one mediated via DNA replication (reviewed in Kogoma 1997; Kowalczykowski 2000; Marians 2000; Michel 2000).

The first experimental evidence supporting the hypothesis that DNA replication is involved in RecBCD-mediated recombination came in 1974 (Siegel 1974). Since then, results from a variety of experimental systems have challenged the exclusivity of break-join mechanism as the only pathway for repairing DSBs (reviewed in Chapter 2). The intimate interplay between DNA recombination and replication was shown in phage T4 (reviewed in Mosig 1998; Kreuzer 2000), implied genetically in *E. coli* (for reviews, see Kogoma 1996; Kogoma 1997; Kuzminov and Stahl 1999; Lombardo and Rosenberg 1999; Lombardo et al. 1999; Kowalczykowski 2000; Marians 2000), yeast (Bosco and Haber 1998; Holmes and Haber 1999; Flores-Rozas and Kolodner 2000) and mammalian cells (for a review, see Harris et al. 1999). In Chapter 2, I directly demonstrated the existence of a replicative DSBR mechanism by isolating recombinant DNA products that have undergone new DNA synthesis from wild-type *E. coli.* I used phage lambda (λ) as the DNA substrate for RecBCD-mediated DSBR and showed that a replicative mechanism occurs in addition to the previously described break-join pathway (see Figure 1). This pathway requires DNA synthesis (break-copy) by DNA Pol III and occurs independently of Holliday junction processing proteins (e.g. in *ruv recG* cells). I estimate that each pathway contributes to approximately half of all DSBR in *E. coli* (Chapter 2).

The involvement of replication in the repair of DSBs has been postulated before (e.g. Smith 1991; Kuzminov 1995; Courcelle et al. 1997), but not much is known about the molecular details of this process. In *E. coli*, the discovery and characterization of a recombination-dependent DNA replication mechanism that operates independently of the origin of replication (*oriC*) provoked new research in this field (reviewed in Kogoma 1997). This type of replication (known as stable-DNA replication, SDR) occurs only in special mutant *E. coli* cells (e.g. *rnhA*) or during the SOS response to DNA damage. Here the requirement of recombination functions in promoting this type of DNA replication has been demonstrated genetically. Of note, the replication primosome assembly protein, PriA, and the strand-exchange recombination protein, RecA, are both required for SDR .

PriA can bind single-strand DNA and direct the assembly of replication forks on DNA intermediates (D-loops) (Liu and Marians 1999; Liu et al. 1999; Marians 2000), and its absence results in a two-thirds reduction in transductional and conjugational recombination (Kogoma et al. 1996). From this result it can be inferred that replicative recombination is responsible for two-thirds of all recombination. However the direct demonstration of a recombined DNA that has also experienced DNA synthesis was not made in this system. Using λ as the DNA substrate, I isolated replicated recombinant DNA molecules, thus demonstrating the existence of a replicative repair pathway in wild-type *E. coli*. Furthermore, I found that this mechanism is responsible for approximately half of all DSBR in *E. coli*.

Interestingly, the RecG protein has been shown to compete with PriA for binding to D-loops in vitro (McGlynn et al. 1997) and the suppressers of *recG* mutation are found in the PriA gene (Al-Deib et al. 1996). D-loops are DNA intermediates formed by the RecA-mediated pairing of a ssDNA to a homologous duplex; these intermediates are hypothesized to be the starting substrate for SDR reactions and to occur during homologous recombination (see Figure 3-1). The finding that PriA competes for binding to a DNA intermediate that occurs normally during recombination allows for the hypothesis that DNA replication may play a role in the processing of HJ recombination intermediates.

We found physical evidence that replicative repair can proceed without the involvement of any HJ processing proteins (Chapter 2). This finding challenges the current models of DSBR in *E. coli*, in which Ruv and RecG systems represent the only pathways of resolving HJ.

In this report, I investigate the role of each HJ processing protein in DSBR in *E. coli*. I use λ DNA as the substrate for recombination (described in Chapter 2), because previously developed methods allow for the easy physical detection, separation and quantification of recombinant progeny formed by each DSBR pathway in vivo. Recombinant progeny are assayed, both physically and genetically, from *E. coli* strains lacking a HJ processing protein. I discover that in *ruvA*, *ruvB*, *ruvC* and *recG* strains, the majority of recombination occurs via the replicative mechanism. The break-join recombinants are either absent (in Ruv-defective strains) or represent a small fraction of total recombination (in *recG* cells). For the first time, these results place RuvABC and RecG in the same (break-join) DSBR pathway.

Interestingly, these results also show that in the absence of RecG protein, the overall recombination frequency remains unchanged (compared to rec^{+}), even though the break-join pathway is hindered significantly. This is in contrast to *ruvA*, *ruvB*, and *ruvC* mutant cells in which overall recombination is reduced to half of wild type. I find that in *recG* cells replicative recombination compensates for this decrease in break-join recombination; however, unlike the previously described break-copy pathway (Chapter 2), it requires RuvABC for completion. These results suggest the occurrence of two distinct DNA intermediates during DSBR in vivo: one requires DNA synthesis by DNA Pol III, but no HJ resolution proteins; the other is processed exclusively by RuvABC, but requires either RecG (break-join) or DNA synthesis (break-copy) to complete the recombination reaction.

Results

λ as a tool to study the RecBCD recombination system in *E. coli*

Bateriophage λ provides a powerful tool for studying the molecular mechanism of RecBCD-mediated recombination in *E. coli.* Its well-established genetics combined with sensitive methods for detecting newly synthesized DNA make it an ideal tool to directly study the connection between DNA recombination and replication in vivo: newly synthesized DNA can be detected on the same molecule that has undergone recombination (reviewed in Stahl 1986; Stahl 1998). Because λ has its own recombination systems, special mutant λ strains, *red gam,* that are defective for λ recombination, are used so that swapping between λ chromosomes only occurs through the *E. coli* RecBCD machinery (Lam et al. 1974; Henderson and Weil 1975).

λ DNA packaging requires dimeric or multimeric units of λ genome connected together in a head-to-tail fashion (λ monomers are not packaged) (for review, see Feiss and Becker 1983). Following λ infection, bi-directional (θ) replication occurs and circular monomeric λ genomes are produced. Normally, multimers are produced via rolling circle replication, requiring the Gam protein. When the Gam protein is absent (e.g. λ *red gam*), rolling circle replication is inhibited because RecBCD degrades rolling circles. Therefore, in RecBCD$^+$ cells, the only route to dimerization and packaging of λ *red gam* DNA is via RecBCD-mediated recombination of monomeric λ chromosomes into multimers. Thus λ *red gam* progeny formation can be used as an assay for *E. coli* recombination (reviewed in Smith and Stahl 1985).

Another advantage of λ is that during packaging a DNA break is made at a defined site called *cos* (Kobayashi et al. 1982; Kobayashi et al. 1983; Stahl 1986). During packaging, *cos* cleavage generates a DSB, which is the substrate for all

RecBCD-mediated events. One end of the DNA is bound by the packaging protein, Terminase, blocking the access of RecBCD enzyme, whereas the other end serves as the entry site for RecBCD. Thus all recombination events are initiated from the same break site.

Because λ packaging is precise, each virion has a defined density. This can be used to detect new DNA synthesis physically (using density labeling techniques described in Chapter 2) while determining the interval of recombination within the same DNA molecule. This technology allows for exploring the interplay between replication and recombination in the *E. coli* RecBCD system. Indeed much mechanistic insight has been gained from the analysis of λ recombination products (e.g. Stahl and Stahl 1971; McMilin and Russo 1972; Stahl et al. 1974; Motamedi et al. 1999).

Recombination in the absence of HJ processing proteins is RecBCD-mediated

Previously, I showed that in cells defective for both Ruv and RecG functions recombination can occur, is RecBCD-mediated, but uses a replicative mechanism (see Chapter 2).

Here I examine the role of each HJ processing protein in RecBCD-mediated recombination in *E. coli*. Using a standard λ recombination assay (modified from Thaler et al. 1989; described in Chapter 2; Razavy et al. 1996) frequency of RecBCD-mediated recombination is measured in mutant *E. coli* strains defective for one HJ processing protein. Briefly, this assay measures the recombination frequency of two λ strains (*red gam*), defective in their own recombination systems (see Figure 3-2A). Because λ *red gam* progeny formation requires recombination (see above for explanation), in order to determine the recombination frequency, an alternative recombination (DNA dimerization) route is provided via the λ site-specific Int recombination system. (Int recombination occurs only at a specific site, *att*, and is independent of the HJ processing proteins of *E. coli*.) This is done because, in this assay, the Int-recombinants can be physically separated from the rest of λ progeny based on their density: they will have the longest piece of DNA, compared to all other recombinants, packaged into a λ capsid (see Figure 3-2A). In a cesium formate density gradient, the Int-recombinants appear in the densest fractions (first peak) and the frequency of gratuitous homologous recombination is measured among them (Chapter 2).

To test whether recombination in cells defective for HJ processing proteins is RecBCD-mediated, I determined the effect of Chi, an octomeric DNA sequence (5' GCTGGTGG 3'), which specifically stimulates RecBCD-mediated recombination in its vicinity (Stahl and Stahl 1977; Ponticelli et al. 1985; West 1992; Kowalczykowski et al. 1994; Myers and Stahl 1994), on λ recombination in this assay. Parallel crosses with Chi^+ and Chi° phages (Figure 3-2A) were performed and the recombination frequency was measured in *rec*$^+$, *ruvA*, *ruvB*, *ruvC*, *recG* and *ruvC recG* cells. Figure 3-2B shows that Chi promotes recombination and its level of activity (recombination frequency in the Chi^+ cross/recombination frequency in the Chi° cross) is the same in all strains tested (Table 3-1, also see Chapter 2 for detailed description). I conclude that recombination in cells defective for HJ processing protein is RecBCD-mediated.

Half of all RecBCD-mediated λ recombination requires RuvA, RuvB, and RuvC proteins

I provided genetical and physical evidence that by removing the HJ processing proteins of *E. coli*, all break-join events are eliminated; the remaining recombination in *ruv recG* cells (half of *rec*$^+$) is replicative (see Chapter 2). Thus, I developed an assay in which the frequency of replicative recombination is measured independently of the break-join pathway by removing the HJ processing proteins. Here I measured the frequency of recombination in *ruvA, ruvB, ruvC*, and *recG* cells and compared these values to the *rec*$^+$ and *ruvC recG* control strains. Figure 3-2B shows that the frequency of recombination is decreased by about half (for both Chi$^+$ and Chi^o crosses) in cells defective for RuvA, RuvB, and RuvC proteins compared to *rec*$^+$. This decrease is also observed for *ruvC recG* cells here (Figure 3-2B) and previously in Chapter 2. I conclude that half of all recombination requires RuvABC and propose that RuvA, RuvB, and RuvC proteins are required for the RecBCD-mediated break-join pathway of recombination in *E. coli*, supporting the hypothesis that these proteins act as a complex in vivo. This is not surprising especially considering that RuvC is the only known *E. coli* protein that specifically cleaves HJs (West 1994; Kuzminov 1996) and its activity requires RuvAB function in vivo (Sharples et al. 1990; Lloyd 1991) and its catalytic function is enhanced by the presence of RuvA and RuvB proteins in vitro (van Gool et al. 1998; van Gool et al. 1999). In experiments below, I directly test the involvement of RuvA, RuvB, and RuvC proteins in break-join recombination by physically examining the recombination products recovered from cells defective for a Ruv protein.

I find that recombination frequency in *recG* cells is approximately the same as *rec*$^+$ for both Chi$^+$ and Chi° sets of experiments (Figure 3-2B). I conclude that the absence of RecG protein has no effect on the overall recombination frequency. This may be interpreted that RecG plays no role in the break-join or break-copy pathways of recombination in *E. coli*. However, direct physical analysis of the recombination products from *recG* cells is required to make any conclusions about the nature of recombination events in this strain. We directly examine the recombination products recovered from *recG* cells in experiments below.

Also, note that recombination drops to half of *rec*$^+$, if any *ruv* mutation is present in *recG* strains (see figure 2-3C and Chapter 2). This suggests that half of all RecBCD-mediated recombination in *recG* cells is RuvABC dependent. Further physical analysis of recombination products from *recG* cells is required to determine the role of Ruv proteins in the absence of RecG.

RecBCD-mediated recombination in *ruvA*, *ruvB*, and *ruvC* cells requires DNA synthesis via DNA Pol III

Recombination in cells defective for both HJ processing systems, *ruvC recG*, is replication-dependent and uses DNA Pol III. A model in which replication helps to process recombination intermediates is presented in Figure 3-1, (modified from Chapter 2, also see Morgan and Severini 1990). Here, λ recombination is examined in the absence of RuvA, RuvB, or RuvC proteins (see Figure 3-2B) and DNA replication. If replication is required for recombination in *ruv* mutant cells, I predict that no recombinant phage should be detected when replication is blocked.

I assayed λ recombination in cells lacking a Ruv protein, which also carry a temperature sensitive mutation in the *dnaE* gene (the polymerase subunit of DNA Pol III). I blocked λ replication by shifting the cells to the restrictive temperature upon infection (see Material and Methods). I used λ that carry two deletions, *bio1* and *nin5*, removing all λ recombination functions, including the Int system and the λ HJ processing protein, Rap (Mandal et al. 1993; Sharples et al. 1994; Mahdi et al. 1996), respectively. This way, the recombination of λ chromosomes can occur only by the *E. coli* recombination proteins and only via the break-join pathway (because replication is blocked). Therefore, in this assay, λ progeny formation is a measure of the recombination proficiency of the *E. coli* strain in the absence of DNA replication.

In order to distinguish between progeny and parental phage, density-labeled λ, grown in the presence of heavy isotopes (^{13}C and ^{15}N), are infected into unlabeled *E. coli* cells. During the lytic infection, all progeny must incorporate light isotopes from the media. In a cesium formate density gradient, progeny phage can be separated from the parental unadsorbed phage based on their densities: parental phage will be heavier (denser) than progeny phage because both their capsid and DNA are made from the heavy isotopes. In contrast, the progeny phage will have heavy DNA (because replication is fully blocked at high temperature) packaged into light protein coats (made from ^{12}C and ^{14}N isotopes). This allows for easy detection of any progeny phage following the lytic infection of λ in different *E. coli* strains (Chapter 2). Also, this assay will show whether a full block to replication has been achieved, because any new DNA synthesis would incorporate light nucleotides which can be detected in a density gradient.

In Figure 3-3, the density gradients of parallel λ infections are graphed. Note that fractions containing particles with the highest density are to the left of each graph. In the *rec*$^+$ graph (Figure 3-3D), two peaks are observed. The first peak from the left represents unadsorbed parental phage (phages that did not infect an *E. coli* cell), carrying heavy protein coats and heavy (HH) DNA. [Their density is identical to that of the labeled phage in the stock used for the experiment (Figure 3-3A)]. They are not part of the λ progeny but can be used as a density reference in the gradient. The next smaller, less-dense peak (in Figure 3-3D) represents phage progeny recovered from the lytic infection. These phage have fully heavy (HH) DNA packaged into light protein coats. Note that the absence of lighter peaks confirms that replication was fully blocked. I infer that these phages are formed following the RecABCD-mediated break-join recombination of monomeric λ chromosomes (McMilin and Russo 1972). In support of this, parallel infection of *recA* (recombination-deficient) strain yields no phage progeny (Figure 3-3B and Chapter 2).

λ infections of *ruvA*, *ruvB*, and *ruvC* cells yield little or no phage progeny when replication is fully blocked (Figure 3-3E, F, G, respectively). From these data, I infer that break-join recombination requires the RuvABC proteins. This is similar to the *ruvC recG* infection in which progeny formation was shown to require DNA replication (Figure 3-3C, and Chapter 2). This suggests that recombination in the absence of any of the Ruv proteins requires DNA synthesis, is replicational. Furthermore, the enzyme responsible for this synthesis is DNA Pol III, because replication was blocked by using a temperature sensitive allele of *dnaE* gene, encoding the replicative subunit of DNA Pol III enzyme.

In the absence of RecG, break-join recombination is significantly reduced compared to *rec*$^+$: evidence that RecG is required for full efficiency of RuvABC

As illustrated in Figure 3-3H, some HH λ recombinant progeny are recovered from *recG* cells, indicating that break-join recombination occurs in the absence of DNA replication; however, the extent of progeny formation is significantly reduced compared to *rec*$^+$ cells. Three sets of independent experiments were performed with *ruvA*, *ruvB*, *ruvC*, *recG* and *rec*$^+$ cells and in each case a progeny peak was observed from the *recG* infections (unlike the Ruv-defective strains), but its size was always smaller than the *rec*$^+$ progeny peak. I measured total progeny recovered from the *rec*$^+$ and *recG* infections by determining the area of the progeny peaks in Figures 3-3D and 3-3H. Even though the size of progeny peaks varied considerably from experiment to experiment, I found that λ progeny formation is reduced (the average of three independent experiments) by roughly 3.5 ± 0.8-fold in *recG* cells compared to the *rec*$^+$ control. I conclude that some break-join recombination occurs in the absence of RecG protein, but its extent is significantly reduced compared to the rec^+ control. (Also note that the *recG* mutation used in this experiment is a null allele.) This implies that RecG is required for the maximum efficiency of RuvABC-dependent break-join recombination. Thus in the absence of RecG and DNA replication, RuvABC-dependent break-join recombination occurs roughly 3-fold less efficiently (Figure 3-3H) than when RecG is present (Figure 3-3B).

Because the overall recombination frequency is unchanged (see Figure 3-2B) and yet there is a significant decrease in break-join recombination in *recG* cells (compared to the rec^+ control), I hypothesize that a replicational mechanism compensates for the decrease in break-join recombination, bringing the overall recombination frequency up to the rec^+ level. The examination of recombination products from *recG* cells will test this hypothesis directly (see below).

RuvA, RuvB, and RuvC proteins are all required for break-join recombination: physical evidence for break-copy recombination in Ruv-defective strains

Recombination in the absence of HJ processing proteins occurs via a break-copy route exclusively (Chapter 2). This replicative mechanism occurs in addition to the break-join pathway in rec^+ *E. coli.* In Figure 3-1, both mechanisms for recombination are depicted. According to the break-copy model, replication copies information from an intact duplex following the pairing of homologous molecules via the RecABC-dependent pathway. Semi-conservative replication of DNA molecules to the end, followed by conservative segregation of the DNA strands (without the use of an endonuclease) would produce recombinant molecules that contain old, parental DNA linked to newly replicated DNA at the site of synthesis. A prediction of this is that central recombinants formed by break-copy recombination would contain half old, parental DNA linked to half newly synthesized DNA; end recombinants would contain mostly old, parental DNA linked to a small track of newly synthesized DNA at the end of the chromosome. This is in contrast to the prediction of a break-join model in which both central and end recombinants would be made entirely from unreplicated parental DNA (see Figure 3-1, and Chapter 2).

I tested these predictions in *ruv* or *recG* strains using a λ recombination assay (described in detail in Chapter 2) in which the interval of recombination and DNA synthesis (incorporating nucleotides from the media) can be assayed simultaneously among progeny phage (adopted from Sawitzke and Stahl 1997). Density-labeled λ (^{13}C and ^{15}N) were infected into unlabeled *E. coli* cells (grown in the presence of light isotopes, ^{12}C and ^{14}N) under conditions that allow for some DNA replication (see Materials and Methods and Chapter 2). The phage carried genetic markers such that recombination in the center and end interval of λ chromosome could be scored separately (Figure 3-4A). Because any new DNA synthesis incorporates light nucleotides, phage progeny carrying new DNA material can be detected in a density gradient. By allowing for some DNA replication to occur, both break-join and break-copy recombinants can be examined among the progeny phage.

A cesium formate density gradient was set up for each cross lysate. Progeny λ were separated from parental unadsorbed phage: as described above, unadsorbed parental phage contain heavy protein coat with heavy DNA and are found in the densest peak of the cesium formate density gradient. Within the progeny class, three peaks appear, based on the extent of new DNA synthesis experienced by each DNA molecule prior to packaging. DNA molecules that experience no (or very little) replication are found in the heavy-heavy (HH) progeny peak. This is followed by heavy-light (HL) and light-light (LL) peaks, containing phage with increasing amounts of newly synthesized DNA. The distribution of central and end recombinants was determined for each fraction of each progeny peak in the gradient.

Chapter 2 provides direct physical evidence for the occurrence of break-join recombination in *rec*$^+$ cells by observing central recombinants under the HH peak of *rec*$^+$ gradient. These central recombinants contain mostly heavy DNA and were formed by the cutting and rejoining of parental (heavy) DNA molecules, without extensive DNA synthesis, a prediction of the break-join model. By contrast, in *ruvC recG* infections, I found few central recombinants (compared to end recombinants) under the HH progeny peak, indicating that break-join recombination, yielding central HH recombinants, does not occur appreciably in the absence of Ruv and RecG proteins. This is consistent with my finding that in the absence of DNA replication, no recombination was observed in *ruvC recG* cells (Figure 3-3D and Chapter 2).

In this chapter, I sought to extend the observations in Chapter 2 by examining the mechanism of recombination in cells defective for each one of the Ruv proteins in turn. In all the graphs depicted in Figure 3-4B, the first peak contains unadsorbed parental phage. They are not part of the λ progeny and only serve as a density reference. In the *rec*$^+$ control infection, I observe HH central recombinants (fractions 34-38), as expected, indicating that break-join recombination occurs normally in *E. coli*. Also note that central (dark circle) recombinants occur more frequently than end (open circle) recombinants. This difference most likely reflects the genetic distance within each interval: central recombinants could arise from recombination within 18-22 kb of DNA (distance from *c*I to *Jts*) whereas end recombinants span approximately 4.8 kb of DNA (distance from *c*I to *S* gene). See Figure 3-4A for an illustration.

If all recombination in Ruv-defective strains is replicational then the HH central recombinants would be absent and occur less frequently than end recombinants, similar to the *ruvC recG* infections (Chapter 2). The remaining panels in Figure 3-4B represent density gradients of progeny phage from *ruvA*, *ruvB*, and *ruvC* infections. I observe almost no central recombinants under the HH peak of *ruvA* (fractions 48-50), *ruvB* (fractions 37-39) and *ruvC* (fractions 33-35) infections; in fact 65-85% of the observed recombinants in this peak (HH) are end recombinants in *ruvA*, *ruvB* and *ruvC* infections. (This is in contrast to the observed frequency of end recombinants under the HH peak of *rec*$^+$ infection (fractions 34-38) in which only approximately 19% of recombinants are end recombinants.)

I conclude that in the absence of any of the Ruv proteins, break-join recombination does not occur significantly and almost all recombination requires DNA synthesis. This is consistent with the previous experiment that in the absence of DNA replication no progeny phage are detected in Ruv-defective strains (see Figure 3-3E, F, G). So, why are there any (end) recombinants under the HH peak in Ruv-defective strains? It is possible that the end recombinants come from a small amount of synthesis at the end of the chromosome (Chapter 2). This small track of new DNA is insufficient to create a significant density change to move these phage from the HH peak (Figure 3-1) and represents roughly 8% of the entire genome.

Figure 3-4B shows that an excess of central to end recombinants occurs under the HL peak of every cross compared to the HH or the LL peaks of the same graph. This result is identical to that seen in the *ruvC recG* double mutant host (Chapter 2) and is expected under the break-copy model (see Figure 3-1 and Chapter 2 for a detailed explanation). According to the model, these recombinants would contain half old, parental DNA and half new, light DNA, banding at the HL peak of the gradient.

Physical evidence that RecG is required for the maximum efficiency of the RuvABC-dependent break-join recombination

In this section, the recombination products from a *recG* cross, using the aforementioned recombination assay, were examined (Figure 3-4C). Central and end recombinants were scored for each fraction of the *rec*$^+$, *ruvC*, *recG*, and *ruvC recG* infection. As discussed and shown above and in Chapter 2, central recombinants were detected and found to occur more frequently than end recombinants under the HH peak of the *rec*$^+$ infection, indicating that break-join recombination occurs normally in these cells (Figure 3-4C). For *ruvC* (fractions 56-58) and *ruvC recG* (fractions 51-53) infections, almost no HH central recombinants were detected, as predicted if all recombination requires DNA synthesis in these strains (Figure 3-4C, also see Chapter 2 and Figure 3-4B).

The results in Figure 3-3H suggest that break-join recombination occurs less efficiently in *recG* cells compared to *rec*$^+$. This is seen as a smaller progeny peak recovered from *recG* infections compared to *rec*$^+$. If RecG is required for optimal break-join recombination, then I predict that fewer HH central (compared to end) recombinants will be observed for the HH peak of *recG* infection compared to the *rec*$^+$. [This can be measured as a ratio of central to end recombinants found under the HH peak for each infection (see Table 3-2).] I detect that central and end recombinants occur with equal frequency for the HH peak of the *recG* infection. This is in contrast to the *rec*$^+$ infection, in which central HH recombinants occur approximately 5 times more frequently than end recombinants under the HH peak (see Table 3-2). (Note that the ratio of HH central to HH end recombinants for the *rec*$^+$ is 4-5 fold higher than the *recG* infection.) Because this ratio can be used as a measure of the efficiency of break-join recombination, this result is consistent with the observation that in the complete absence of DNA replication, a smaller-than-*rec*$^+$ progeny peak is recovered from *recG* infections (compare Figures 3-3D, and 3-3H).

These data (Figure 3-4C) together with the data presented in Figure 3-3 indicate that RecG is required for the full efficiency of the RuvABC-dependent break-join recombination: in its absence fewer break-join recombinants are recovered (compare Figures 3-3D and 3-3H) and break-join recombination occurs less efficiently than *rec*$^+$ (see Figure 3-4C). Despite this decrease in break-join recombination, I find that the overall frequency of recombination in *recG* cells remains unaffected (see Figure 3-2B). I propose that the majority of recombination in the absence of RecG is mediated via replication. In support of this, I observe the accumulation of central recombinants under the HL peak of the graph, indicating that the central recombinants that are not resolved by the break-join pathway are resolved via replication to the end of the chromosome.

Absence of any of the HJ processing proteins promotes replication of λ dramatically

As shown in Chapter 2, the absence of RuvC and RecG proteins enhances replication dramatically compared to the *rec*$^+$ control. Here I find the same phenotype for cells defective for any one of the HJ processing proteins. This can be determined by calculating the area under the LL peaks of *ruvA*, *ruvB*, *ruvC* and *recG* experiments and comparing them to the LL peak of the corresponding *rec*$^+$ control. I observe few or no LL progeny from *rec*$^+$ infections (Figure 3-4B, C), in contrast to the large LL progeny peak derived from HJ-defective hosts. This indicates that the accumulation of recombination intermediates, caused by the absence of any of the HJ processing proteins, promotes replication dramatically.

Discussion

Previously, I showed that the RecBCD system of DSBR recombination in *E. coli* operates via two parallel mechanisms: one requires DNA synthesis (break-copy) via DNA Pol III, but not the HJ processing proteins, while the other requires HJ processing proteins, but occurs independently of DNA synthesis (break-join) (see Chapter 2 and Figure 3-1). I hypothesized that both mechanisms occur simultaneously, processing recombination intermediates into mature recombinant products in wild-type *E. coii*. In Chapter 2, the predictions of this model were tested by examining the extent of new DNA synthesis associated with the formation of recombination products. I found that both types of recombination product are recovered from *rec*$^+$ cells and each mechanism contributes to approximately half of all RecBCD-mediated DSBR in *E. coli*. In cells defective for HJ processing (*ruv*C *rec*G), we found that break-join recombination is eliminated, recombination frequency is reduced to half of wild type, and essentially all remaining recombination requires DNA replication (Chapter 2).

The discovery of a recombination mechanism operating via DNA replication and independently of the HJ processing proteins was unique because of the widely accepted notion that all recombination events in wild-type *E. coli*, under normal conditions, are processed by the HJ processing proteins (e.g. Thaler and Stahl 1988; Kowalczykowski et al. 1994; Myers and Stahl 1994; Kowalczykowski 2000). In this chapter, I examined the individual roles of each HJ processing protein in the RecBCD-pathway of DSBR in vivo.

The analysis revealed the following:

1. In the absence of RuvA, RuvB, or RuvC, proteins, half of all RecBCD-mediated λ recombination is eliminated (Figure 3-2B). The remaining half requires DNA replication via DNA Pol III (Figure 3-3).

2. Replicative recombination was demonstrated directly by the physical analysis of recombination products from Ruv-defective strains. Essentially all break-join recombination events are eliminated as revealed by the absence of central recombinants with HH DNA (Figure 3-4). New DNA synthesis was almost always observed with central recombinants found in excess (relative to end recombinants) under the HL progeny peak, demonstrating a replicative origin for their formation.

3. *ruvA*, *ruvB*, and *ruvC* strains display the same phenotype in our recombination assays (Figures 3-2B, 3-3, 3-4B), supporting the hypothesis that the three proteins form a complex (resolvasome) in vivo (Kuzminov 1996; Eggleston et al. 1997; van Gool et al. 1999).

4. In the absence of RecG, the overall recombination frequency remained unchanged compared to the *rec*$^+$ control (unlike Ruv-defective strains, see Figure 3-2B), however we found a significant decrease in break-join recombination in these cells (Figures 3-2B, 3-3, 3-4C). This drop in break-join recombination was not as severe as the ones seen in Ruv-defective strains. Furthermore, the physical analysis of recombination products from *recG* cells for break-join recombination revealed that break-join recombination occurs about 3-5 fold less efficiently in these cells than the *rec*$^+$ control (Figures 3-3, 3-4C). These data indicate that the RuvABC and RecG proteins are required for the optimal operation of RecBCD-mediated break-join recombination: in the absence of RecG, fewer break-join recombinants are observed.

Even though there is a significant decrease in break-join recombination, I explain the unreduced overall recombination frequency in *recG* cells by suggesting that a novel RuvABC-dependent replicational mechanism compensates for the decrease in break-join recombination in *recG* cells. In support of this, I found an excess of central recombinants under the HL progeny peak, indicating that the majority of these recombinants are formed by a replicative mechanism. Unlike the previously described replicative recombination pathway, which does not require any HJ processing proteins, the one proposed in *recG* cells, that compensates for the decrease in break-join recombination, requires the RuvABC proteins (Figure 3-2C). In support of this, I found that in *ruv recG* cells, the overall recombination frequency drops to half of wild type and almost all break-join recombination is eliminated (Chapter 2 and Figures 3-2C, 3-4C).

5. I found that the absence of any of the HJ processing proteins enhances replication dramatically. A large LL progeny peak was detected in the *ruv* or *recG* infections, but this was absent in the *rec*$^+$ infection. I propose the absence of any of the HJ processing proteins results in the accumulation of recombination intermediates, which could serve as DNA sites for PriA-directed replication fork assembly. Replication is thus enhanced as more assembly sites accumulate in cells defective for HJ processing.

The data discussed above provide physical evidence that Ruv and RecG, previously thought to be independent pathways for processing recombination intermediates (Lloyd 1991), work together and are required for the optimal efficiency of break-join recombination in *E. coli*. The data are summarized graphically in Figure 3-5 and argue for the occurrence of two distinct recombination intermediates: one that requires processing via RuvABC, and one that is processed independently of RuvABC or RecG, and uses a replicative mechanism (see below for a model). I propose that RecG is required to stabilize recombination intermediates (by perhaps extending the heteroduplex DNA region via branch migration the HJ to the left) for processing via RuvABC such that in its absence, the RuvABC-mediated break-join pathway operates at a lower efficiency.

Model for the existence of two distinct recombination intermediates

The data in this and Chapter 2 can be reconciled by proposing that two distinct DNA intermediates occur during recombination in vivo (see Figure 3-5 data summary). I hypothesize that each intermediate has a genetically distinct pathway for maturation: one ("A") intermediate requires processing via the RuvABC proteins, while the other ("B") requires neither the RuvABC nor the RecG, but is processed via a replicative mode, requiring DNA Pol III (see Figure 3-6).

According to this model, RecG and the replication primosome assembly protein, PriA, compete for binding to the same ("A") DNA intermediate in vivo. RecG and PriA proteins have been shown directly to compete for binding to the same DNA substrate (D-loops) in vitro (McGlynn et al. 1997) and indirectly in vivo (Al-Deib et al. 1996). I hypothesize that the outcome of this competition determines the mechanism employed for processing of the intermediate (see Figure 3-6 for details). Although, PriA can bind to either intermediate, I propose that in the presence of RecG, intermediate "A" is processed mostly via the RuvABC-dependent break-join pathway. In the absence of RecG, PriA can bind to "A" more frequently and break-join recombination is reduced by 3-5 fold. The ensuing DNA Pol III-dependent replication extends the recombination intermediate, but this reaction still requires RuvABC for completion (recombination in *ruv recG* cells is half of *rec*$^+$). Intermediate B, on the other hand, is processed by a replicative route, requiring DNA Pol III, independently of any HJ processing proteins.

The evidence that supports the two-intermediate hypothesis is that in *ruv recG* cells, in the absence of competition between RecG and PriA, I observe that overall recombination drops to half of *rec*$^+$, implying that half of all events are absolutely RuvABC-dependent. The postulated PriA-dependent replicative recombination pathway, which operates on "B", cannot substitute for all recombination in the cell. If this were not the case, then recombination in *ruv recG* cells should be all replicative and equal to *rec*$^+$. Based on our results, it seem plausible that one type of recombination intermediate is RuvABC-specific and the other is independent of RuvABC, but requires DNA replication.

Two types of recombination intermediates: strand polarity?

The data presented above support a model in which two distinct recombination intermediates occur in vivo. These experiments were not designed to determine the strand polarity of the invading end; however, it is possible that the two recombination intermediates represent two invading ends (3' and 5'). This idea was proposed by Rosenberg and Hastings (1991) and supported by Hagemann and Rosenberg (1991), Taylor and Smith (1995), Miesel and Roth (1996) and Shan et al (1997). In vitro results have shown the ability of RecA protein to catalyze strand-exchange recombination reactions using both 3' and 5'-ended strands (Dutreix et al. 1991).

Because not much is known about the structure of the DNA intermediates generated during recombination in vivo, I can not exclude that the postulated recombination intermediates, "A" and "B", are formed via 3'-ended invasions only. That only 3'-ended invasions are recombinogenic is supported by numerous in vitro experiments and in a unimolecular recombination reaction in vivo (Friedman-Ohana and Cohen 1998). Even though my results do not directly address the polarity question, they do suggest the formation of two recombination intermediates in vivo. Further work is required to directly test whether the two types of intermediates are formed based on strand polarity of the invading single strand DNA or other factors involved in the formation of the recombination intermediates (such as other proteins that may play some role in this process, e.g. RecF, RecR, RecO, etc.).

Data from a recombination-dependent stationary phase mutagenesis mechanism operating in *E. coli* combined with the results presented here may have some bearing on the polarity question (for reviews, see Lombardo and Rosenberg 1999; Lombardo et al. 1999). In this system, a RecABCD- DNA Pol III-dependent mutagenesis mechanism operates by creating +1 frame-shift mutations on an F' plasmid, carrying a -1 *lacZ* frame-shift mutation (Harris et al. 1994). A replicative recombination mechanism could account for these results (Foster et al. 1995; Harris et al. 1997). But, importantly, in this system *recG* and *ruvABC* mutations exhibit different phenotypes (Foster et al. 1996; Harris et al. 1996). The Ruv proteins are required for this process whereas RecG is inhibitory. Furthermore, genetical evidence demonstrates the involvement of only 3' DNA ends in the mutagenic pathway (Ross et al. unpublished results). If true, then perhaps this mutagenic pathway represents the RuvABC-dependent mechanism i.e. intermediate "A". Thus, in *rec*$^+$ cells, RecG and the replication primosome assembly protein, PriA, compete for binding to the proposed recombination intermediate "A" formed during this process. If PriA binds, then Pol III directed synthesis of the recombination intermediate may lead to a -1 frame-shift mutation that requires resolution via RuvABC. If RecG binds, those recombination intermediates are resolved without the involvement of DNA synthesis and therefore do not result in the formation of *lac*$^+$ colonies. In the absence of RecG, PriA-directed replication of the proposed intermediate occurs more frequently and more *lac*$^+$ colonies are recovered. The recombination-dependent stationary phase mutagenesis and the results of this chapter taken together imply 3' invasions may be RuvABC-specific.

Transductional and conjugational recombination in *E. coli* and the role of Ruv and RecG proteins in their formation

Using λ as the DNA substrate for RecBCD-mediated recombination, the data presented here and previously demonstrate that in the absence of both Ruv and RecG proteins recombination still occurs, but uses a replicative mode. This is in contrast to other assays for recombination in *E. coli*: *ruv recG* double mutant strains are recombination-defective for conjugational and transductional recombination (Lloyd 1991). This discrepancy in results obtained from different recombination assays has been addressed previously and a general model was presented (Chapter 2). The replication forks initiated at recombination intermediates are structurally different than the those initiated at replication origins such that a HJ is behind the advancing replication fork. If replicative recombination of a linear piece of DNA, the substrate for conjugational and transductional recombination, into the *E. coli* chromosome requires replication (see Kogoma 1997), it is possible that RuvAB or RecG proteins function to branch migrate the HJ (via their helicase activity) around the entire chromosome (4.5 Mb) in order to complete the recombination event. In contrast, the λ chromosome is shorter (48 kb) and branch migration around its chromosome may present a much more manageable topological task. Moreover, a λ-specific function may substitute for the proposed role of RuvAB or RecG proteins in *E. coli*. For example, as discussed before, the packaging machinery of λ, moving in the same direction as the replication fork, may physically push the junction along the replication path assisting in its resolution by driving it past the next packaging origin, *cos* (Chapter 2). This would create a packagable, replicated, recombinant progeny phage. A detailed discussion of the model is presented in Chapter 2. While other explanations of the results are possible, the model presented here makes testable predictions. Moreover, determining the potential role of other recombination proteins such as RecF, RecR, RecO, and RecN, in the context of the two intermediate model, could greatly facilitate the understanding of the molecular details of recombination in the RecBCD system.

Materials and methods

Bacterial and phage strains

All *E. coli* strains used in this paper are K-12 derivatives and are listed in Table 3-3. Standard P1-mediated transduction was used to construct new strains (Miller 1992). SMR650, 3124, 4594, 4600, 4601, 3731, 3732, were constructed as described in Chapter 2. The presence of mutations in recombination genes *recA, ruvA, ruvB, ruvC, and recG* was confirmed by the increase in sensitivity to ultraviolet (UV) light observed for cells with these mutations. SMR5639, 5640, 635, 636 were constructed by transduction of *ruvA200 eda-51*::Tn*10*, *ruvB9 zea-3*::Tn*10*, *ruvC53 eda-51*::Tn*10* and *recG258*::Tn*10minikan* from SMR1549, SMR1552, (Harris et al. 1996),CS85 (Lloyd 1991) and RDK2655 (obtained from R. Kolodner via Lloyd and Buckman 1991) into SMR632, respectively. SMR5641, 5642 were constructed by transduction of *ruvA200 eda-51*::Tn*10*, *ruvB9 zea-3*::Tn*10*, *ruvC53 eda-51*::Tn*10* and *recG258*::Tn*10minikan* from SMR1549, SMR1552, (Harris et al. 1996),CS85 (Lloyd 1991) and RDK2655 (obtained from R. Kolodner via Lloyd and Buckman 1991) into SMR4594, respectively. SMR4287, 4288, 5643, and 5644 were constructed by transduction of *ruvA200 eda-51*::Tn*10*, *ruvB9 zea-3*::Tn*10*, *ruvC53 eda-51*::Tn*10* and *recG258*::Tn*10minikan* from SMR1549, SMR1552, (Harris et al. 1996),CS85 (Lloyd 1991) and RDK2655 (obtained from R. Kolodner via Lloyd and Buckman 1991) into SMR3700, respectively, followed by their lysogenization with λ*Jts15 red3 gam210 Δnin5 Sam7* (Sawitzke and Stahl 1997).

All λ strains used in this report have been described previously in Chapter 2 or referenced therein.

Growth of phage stocks and *E. coli* cultures

All phage and *E. coli* strains were grown according to protocols in Chapter 2, or referenced therein. Strains carrying mutations in *ruvA, ruvB, ruvC, recG* or any combination of the double *ruv recG* mutations were grown at 32° to avoid the accumulation of suppressers normally seen if these strains are grown at higher (37°) temperature (Harris et al. 1996). The UV sensitivity phenotypes for *ruvA, ruvB, ruvC, recG* or *ruv recG* cultures were checked by either directly testing the culture used in the experiment and/or testing approximately 30-40 colonies grown from each culture. (The antibiotic resistance of each culture was also tested.) λ light and heavy stocks were grown as described in Chapter 2 or reference within. Standard plaque assay tests were performed as described in (Murray 1983).

Determination of recombination frequency

As described previously (Thaler et al. 1989; Razavy et al. 1996, and Chapter 2), λ crosses were performed to quantify the frequency of recombination in RecBCD$^+$ cells. Strains were grown to log phase prior to λ infection according to protocols in Chapter 2. Crosses were carried out according to procedures in Chapter 2 or referenced therein. For each cross lysate, a cesium formate density gradient was prepared, spun, and collected as two drop fractions into TB buffer. Titer of phages in each fraction was determined on appropriate hosts as described in Chapter 2.

λ recombination assay in the absence of DNA replication

E. coli cells carrying a temperature sensitive mutation in the polymerase subunit (*dnaEts486*) of DNA Pol III were infected with density-labeled (^{13}C, ^{15}N) λ at the non-permissive temperature of 43.5°. We were able to achieve a full block to replication under these conditions (Chapter 2). The assay was performed as described previously (Chapter 2). Phage titer was determined for each fraction by plating an appropriate dilution on SMR423.

Assay for determining the interval of recombination when replication is partially blocked

These experiments (Figure 3-4B, C) were performed as described in Chapter 2. Note that again cells were grown to log phase at 32° to avoid the accumulation of suppresser mutants. The protocol was followed exactly as before (Chapter 2, modified from Sawitzke and Stahl 1997), except that the gradient was collected as one-drop fractions into 1 ml of TB. Each fraction was titered on JAS38 and JAS36 for recombinants (see Table 3-3).

Table 3-1. The measure of Chi activity in cells defective for HJ processing

	Percent homologous recombination[b] Chi$^+$ (mean ± SD)		Percent homologous recombination[b] Chi^o (mean ± SD)		Chi activity[c] (Chi$^+$/Chi^o)	
E. coli Strains[a]	Expt #1	Expt #2	Expt #1	Expt #2	Expt #1	Expt #2
rec$^+$	21.2 ± 5.0	21.3 ± 3.2	5.0 ± 0.9	6.0 ± 0.6	4.2	3.6
ruvA	11.1 ± 1.9	8.8 ± 1.5	3.2 ± 0.5	2.8 ± 0.5	3.5	3.1
ruvB	10.4 ± 3.0	9.9 ± 1.3	2.9 ± 0.9	3.1 ± 0.4	3.6	3.2
ruvC	8.7 ± 1.5	8.8 ± 0.9	3.3 ± 0.3	3.4 ± 0.8	2.6	2.6
recG	24.1 ± 2.0	26.6 ± 2.7	6.7 ± 1.2	8.3 ± 0.9	3.6	3.2
ruvC recG	10.4 ± 1.3	9.2 ± 1.4	2.8 ± 0.6	3.1 ± 0.5	3.7	3.0

[a] These strains are isogenic derivatives of SMR 632 (Table 3-3).

[b] Percentages of homologous recombination are calculated as described in Figure 3-2 and Chapter 2. Chi$^+$ and Chi^o crosses were performed as described in Figure 3-2A.

[c] Chi activity was measured as described in Chapter 2. Briefly, recombination frequency measured in Chi$^+$ crosses is divided by recombination frequency measured in Chi^o crosses.

Table 3-2. Ratio of central to end (C/E) recombinants measured for the HH peaks of various infections

	Ratio of C/E recombinants[b] for the HH peak		Fold decrease[c] in C/E ratio relative to *rec*+	
E. coli strains[a]	Expt #1[d]	Expt #2[e]	Expt #1	Expt #2
rec+	5.2 ± 1.6	4.7 ± 1.3	1.0	1.0
ruvC	0.16 ± 0.4	0.19 ± 0.6	33	25
recG	0.96 ± 0.3	1.1 ± 0.4	5.4	4.3
ruvC recG	0.33 ± 0.7	0.31 ± 0.4	16	15

[a] All strains are isogenic derivatives of parent strains SMR632 (see Table 3-3 and Materials and Methods for details.

[b] Ratio of central to end (C/E) recombinants was measured for each fraction of the HH peaks of each cross. Mean ratio of C/E for each HH peak was determined ± SD.

[c] Fold decrease in the ratio of C/E recombinants in HH peaks was measured by dividing the mean C/E ratio of *rec*+ cross by the mean C/E ratio for each tested strain. This ratio indicates the distribution of recombinants under the HH peak and can be used as a measure for the efficiency of break-join pathway. A 4- to 5-fold drop in C/E ratio is observed for *recG* cells.

[d] Data not graphed.

[e] Data from Figure 3-4C.

Table 3-3. Bacterial strains

Strain	Relevant Genotype	Source or reference
AFT 196	C600 *Δ(srlR-recA)306*::Tn*10*	Chapter 2
KR3a	SuIII^{+} *recA*	Chapter 2
RDK2641	*ruvA59*::Tn*10*	R. Kolodner
CS85	*ruvC53 eda-51*::Tn*10*	R. G. Lloyd, via Kolodner
RDK2655	*recG258*::Tn*10minikan*	R. Kolodner
SR2210	*ruvA200 eda-51*::Tn*10*	Sargentini via Lloyd via Kolodner
RDK1873	*ruvB9 zea-3*::Tn*10*	R. Kolodner
SMR432	C600 SuII III *recD1903::Tet hsdrK*$^{-}$ *mK*$^{+}$	Lab collection
SMR632	594 *hsdrK*$^{-}$ *mK*	Lab collection
SMR635	SMR632 *ruvC53 eda-51*::Tn*10*	This work
SMR636	SMR632 *recG258*::Tn*10minikan*	This work
SMR650	SMR632 *ruvC53 eda-51*::Tn*10* *recG258*::Tn*10minikan*	Chapter 2
SMR3124	SMR632 *ruvA59*::Tn*10* *recG258*::Tn*10minikan*	Chapter 2
SMR5639	SMR632 *ruvA200 eda-51*::Tn*10*	This work
SMR5640	SMR632 *ruvB9 zea-3*::Tn*10*	This work
SMR4594	SMR632 *dnaEts486 zae*::Tn*10d-cam*	Chapter 2
SMR4595	SMR632 *dnaEts486 zae*::Tn*10d-cam* *ruvC53 eda-51*::Tn*10*	This work
SMR4597	SMR632 *dnaEts486 zae*::Tn*10d-cam* *recG258*::Tn*10minikan*	This work
SMR4600	SMR632 *dnaEts486 zae*::Tn*10d-cam* *ruvC53 eda-51*::Tn*10* *recG258*::Tn*10minikan*	Chapter 2
SMR4601	SMR632 *dnaEts486 zae*::Tn*10d-cam* *Δ(srlR-recA)306*::Tn*10*	Chapter 2
SMR5641	SMR632 *dnaEts486 zae*::Tn*10d-cam* *ruvA200 eda-51*::Tn*10*	This work
SMR5642	SMR632 *dnaEts486 zae*::Tn*10d-cam* *ruvB9 zea-3*::Tn*10*	This work
SMR3700	SMR632 *grpD55 malF*::Tn*10*::*kan*	Chapter 2
SMR3731	SMR632 *grpD55 malF*::Tn*10*::*kan* (*λJts15 red3 gam210 Δnin5 Sam7*)	Chapter 2
SMR4287	SMR632 *grpD55 malF*::Tn*10*::*kan* *recG162 zib-636*::Tn*10* (*λJts15 red3 gam210 Δnin5 Sam7*)	This work
SMR4288	SMR632 *grpD55 malF*::Tn*10*::*kan* *ruvC53 eda-57*::Tn*10*::*cam* (*λJts15 red3 gam210 Δnin5 Sam7*)	This work
SMR3732	SMR632 *grpD55 malF*::Tn*10*::*kan* *ruvC53 eda-57*::Tn*10*::*cam* *recG162 zib-636*::Tn*10* (*λJts15 red3 gam210 Δnin5 Sam7*)	Chapter 2

SMR5643	SMR632 *grpD55 malF*::Tn*10*::*kan ruvA200 eda-51*::Tn*10* (λ*Jts15 red3 gam210* Δ*nin5 Sam7*)	This work
SMR5644	SMR632 *grpD55 malF*::Tn*10*::*kan ruvB9 zea-3*::Tn*10* (λ*Jts15 red3 gam210* Δ*nin5 Sam7*)	This work
JAS36	C600 (λ*Jts15 red3 gam210 imm434* Δ*nin5 Sam7*)	(Sawitzke and Stahl, 1997)
JAS38	*Δ(srlR-recA)306*::Tn*10 recD1009* (λ*Jts15 red3 gam210 imm434* Δ*nin5 Sam7*)	(Sawitzke and Stahl, 1997)

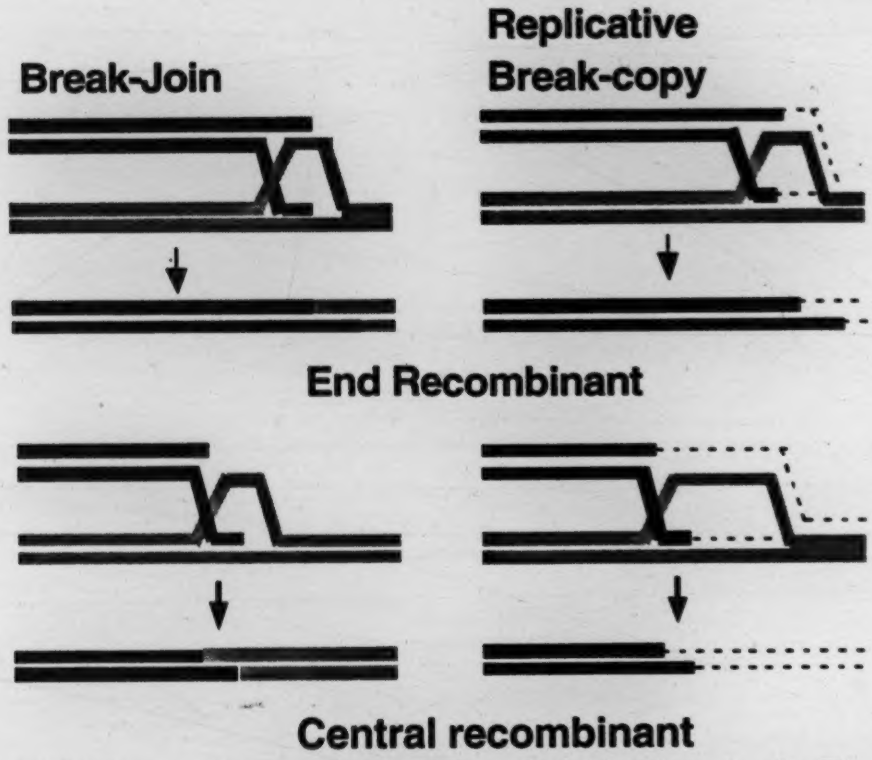


Figure 3-1. Two mechanisms for RecBCD-mediated DSBR in *E. coli*. The thick solid lines represent old, parental DNA; the thin dashed lines represent newly replicated DNA. Following the RecABCD-dependent synapse of homologous molecules, DSBR can proceed via two parallel mechanisms: break-join or replicative (break-copy). (*A*) The break-join process occurs via the cutting and rejoining of homologous molecules at recombination junctions without the involvement of any new DNA synthesis. This process forms both central and end recombination products that are made entirely from parental (thick lines) DNA. (*B*) The replicative (break-copy) mechanism involves the copying of information from an intact duplex via replication to form a recombinant molecule, followed by the conservative segregation of the newly synthesized DNA strands (see Kogoma 1997; Chapter 2). This process forms central recombinants that carry roughly half newly synthesized DNA and half old, parental DNA; the end recombinants carry mostly parental DNA linked to a short track of newly synthesized DNA. In experiments where old parental DNA is labeled with heavy isotopes, central recombinants formed by the break-join and replicative mechanisms can be separated from each other based on their density in a density gradient. The replicative central recombinants will band in the lighter fractions of the gradient than the break-join recombinants. End recombinants from both break-join and replicative mechanisms will band under the HH peak, because a small change in the density of the DNA (as a result of incorporating light nucleotides) is not detected in our assay.

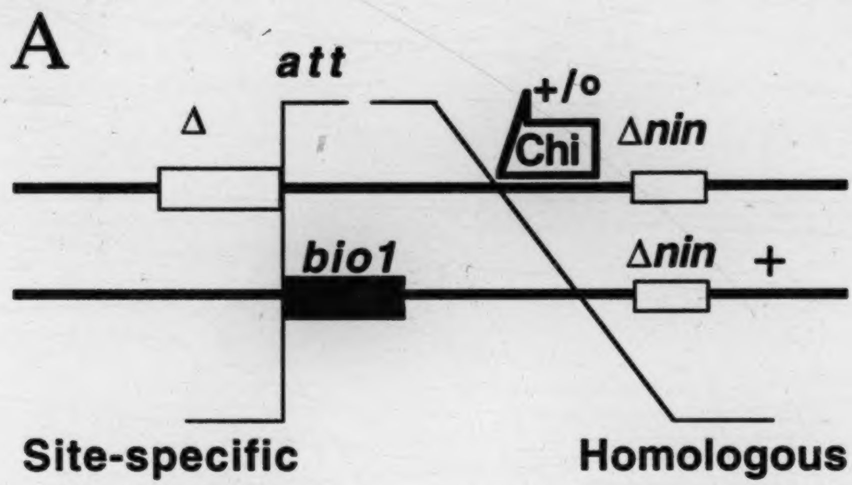
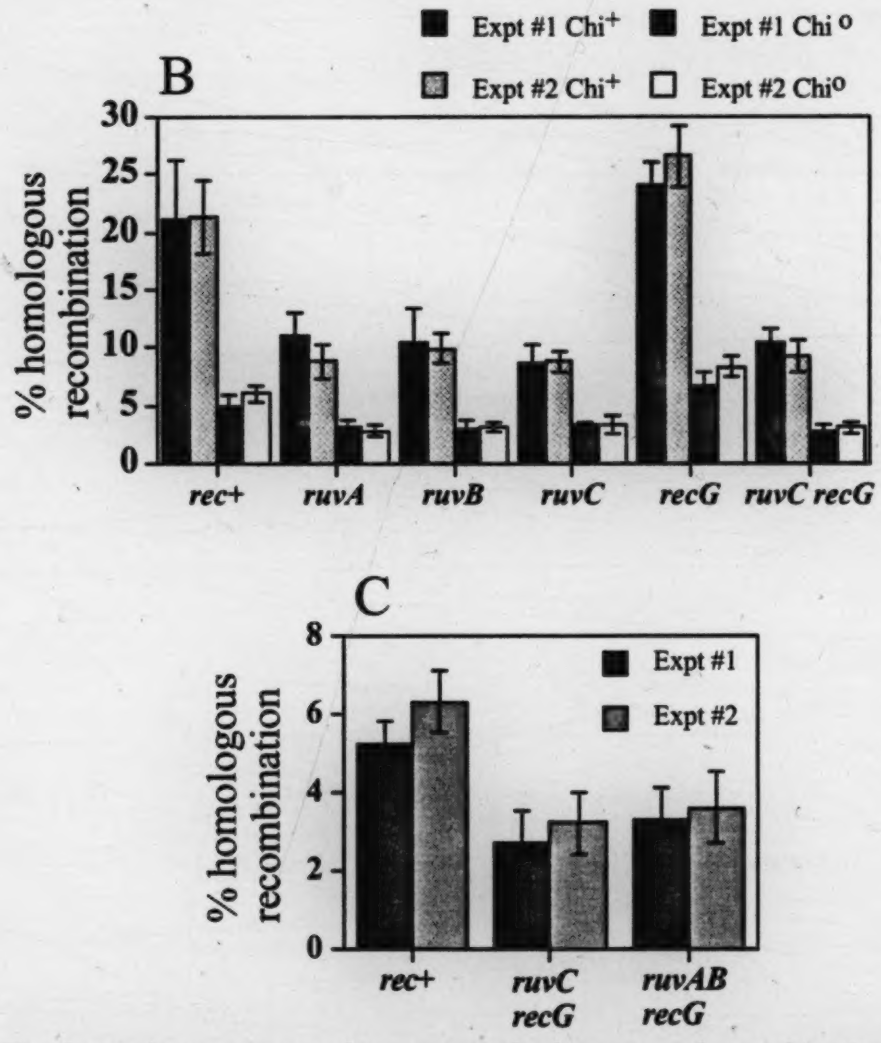
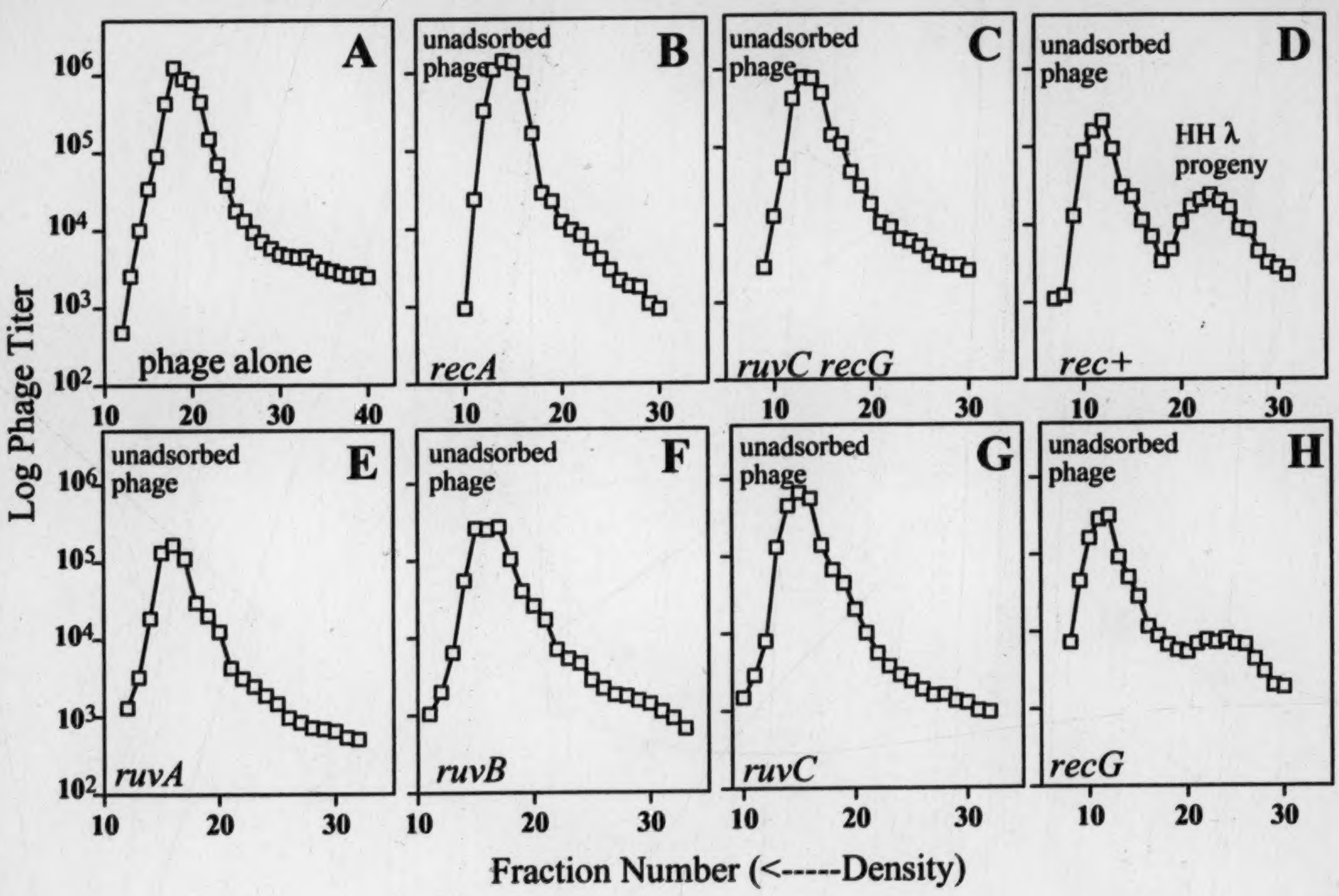


Figure 3-2. RecBCD-mediated recombination of λ *red gam* in cells defective for Ruv or RecG proteins. (*A*) Relevant genotype of phages used in this recombination assay are depicted here and Chapter 2. Open boxes represent deletions; the top left open box represents a deletion (Δ*b527*), removing DNA from the center of *att* site to its left. The open boxes on the right side of both phages represent a deletion (Δ*nin5*) that removes nine open reading frames, including a λ HJ resolving protein (Sharples et al, 1998). In this assay, and all others described in this paper, phages used carry this deletion (Δ*nin5*) so that the processing of recombination intermediates will be done exclusively via the *E. coli* proteins. Also, all phages in this and other experiments in this paper are *red gam*. In this assay, the top phage carries *red3* and *gam210* mutant alleles, whereas the bottom phage carries a deletion/substitution (solid box, Δ*bio1*) that removes λ DNA from the center of *att* site to its right, including *red* and *gam* genes. Two different genotypes of the top phage were used differing only in the presence of Chi, ChiC, in their DNA sequence. Note that from among all recombinants, the Int-promoted events (shown as site-specific) form the longest piece of DNA. The Int-recombinants are isolated from the rest of λ progeny based on their density (see text), and gratuitous homologous recombination is measured among them.



(*B*) Above phages were used to measure the frequency of recombination in cells defective for Ruv or RecG proteins. (The *recG* mutations used is a null allele.) The results from two sets of independent Chi$^{+/o}$ crosses are summarized in this bar graph. Each bar represents results from a different density gradient. In total, the frequency of recombination was measured four times for each strain from two sets of Chi$^{+/o}$ experiments. The value for each bar is the mean percentage of homologous recombination among site-specific Int-mediated recombinants ± standard deviation (shown as error bars). (*C*) The frequency of recombination for *ruvAB recG* strains was determined using the above assay. Only Chi^{o} crosses were performed and the results are presented in this graph. The *ruvA* allele used in this experiment (*ruvA59*) is polar on *ruvB*, therefore, the genotype of the strain is presented as *ruvAB*.

Figure 3-3. λ progeny formation in the absence of Ruv or RecG proteins when replication is blocked. Density-labeled λ *red gam Δnin5* (λSR27; Motamedi et al, 1999) was infected into unlabeled RecBCD$^+$ *E. coli* so that λ progeny formation can be used as an assay for *E. coli* recombination. All strains carry a temperature sensitive allele of *dnaE* gene (*dnaEts486*) and the experiments were performed at 43.5° in order to achieve a full replication block. Following λ infection, lysates were collected and centrifuged in a cesium formate density gradient. Each data point in the graphs represents the titer of λ in the corresponding fraction of the density gradient. Fractions containing phage with the highest density occur to the left of each graph. The first peak (from the left) represents unadsorbed λ. These phage carry heavy protein coats and HH DNA, therefore, have the highest density in the infection lysate. They are not part of the λ progeny, but serve as a density marker. (*A*) This is the density gradient of our heavy phage stock used in the experiment. These phages are centrifuged directly in a density gradient without infecting *E. coli*. The other panels display the density profiles of progeny phage produced in (*B*) a *recA* strain. Because *recA* cells are recombination defective, no progeny phage were detected (negative control). (C) a *ruvC recG* strain. As shown previously, few or no progeny phage are detected. (*D*) a *rec*$^+$ strain. The peak in fractions 22-26 contains phages that have infected the cell, recombined and packaged. They carry light protein coats and HH DNA. Note that no other peaks appear, confirming that a full block to replication was achieved. (*E*) *ruvA* strain. Few or no phage progeny are detected. (*F*) *ruvB* strain. Few or no phage progeny are detected. (*G*) *ruvC* strain. No progeny phage are detected. (*H*) *recG* strain. A small but significant and reproducible progeny peak is detected. Note that the *recG* mutation used in this experiment is a null allele.


A
phage alone
B
unadsorbed phage
recA
C
unadsorbed phage
ruvC recG
D
unadsorbed phage
HH λ progeny
rec+
E
unadsorbed phage
ruvA
F
unadsorbed phage
ruvB
G
unadsorbed phage
ruvC
H
unadsorbed phage
recG
Log Phage Titer
10^6
10^5
10^4
10^3
10^2
10
20
30
40
Fraction Number (<-----Density)

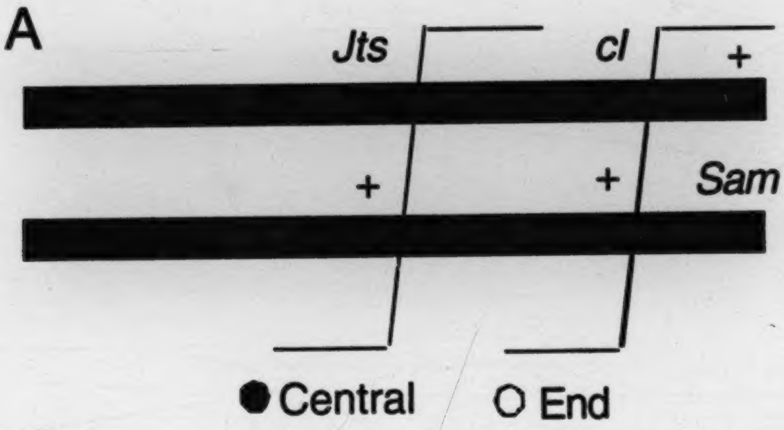
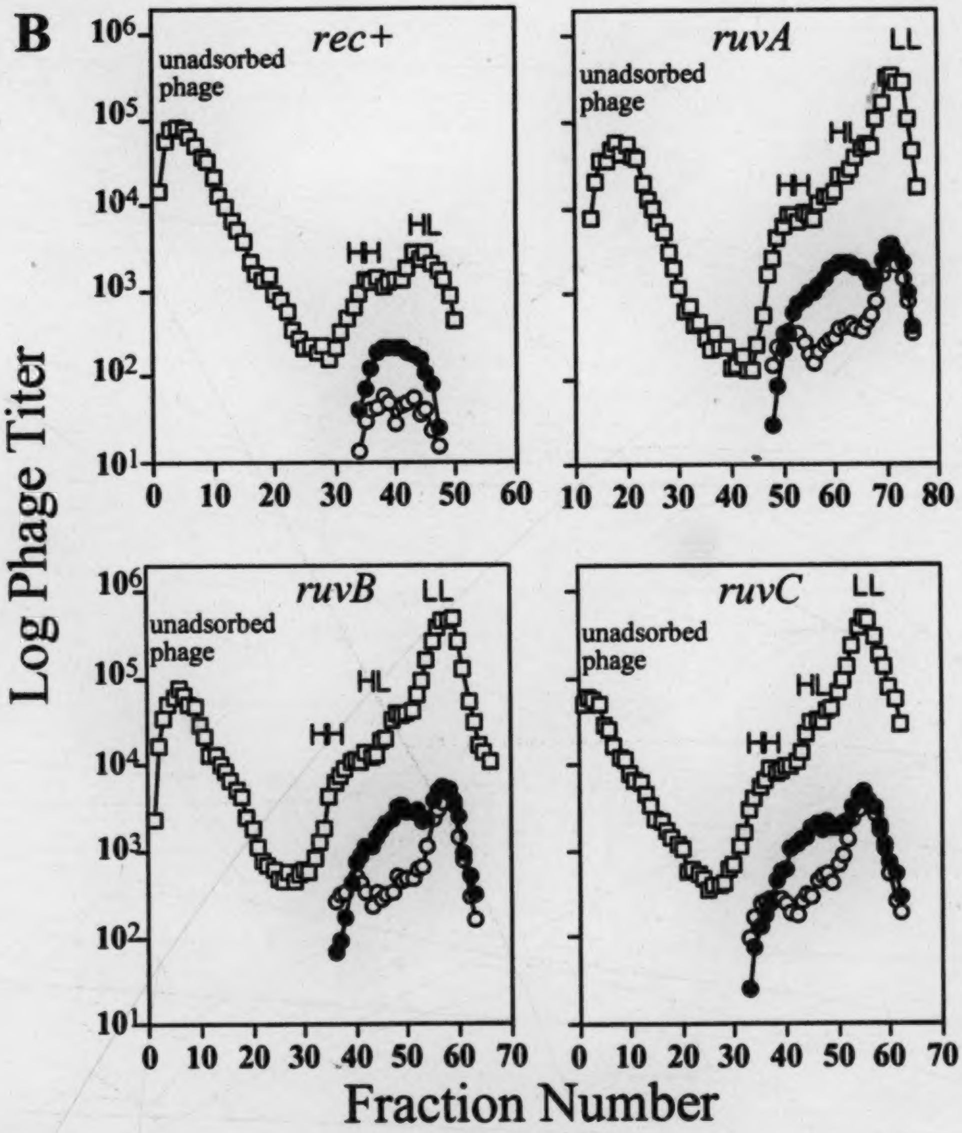
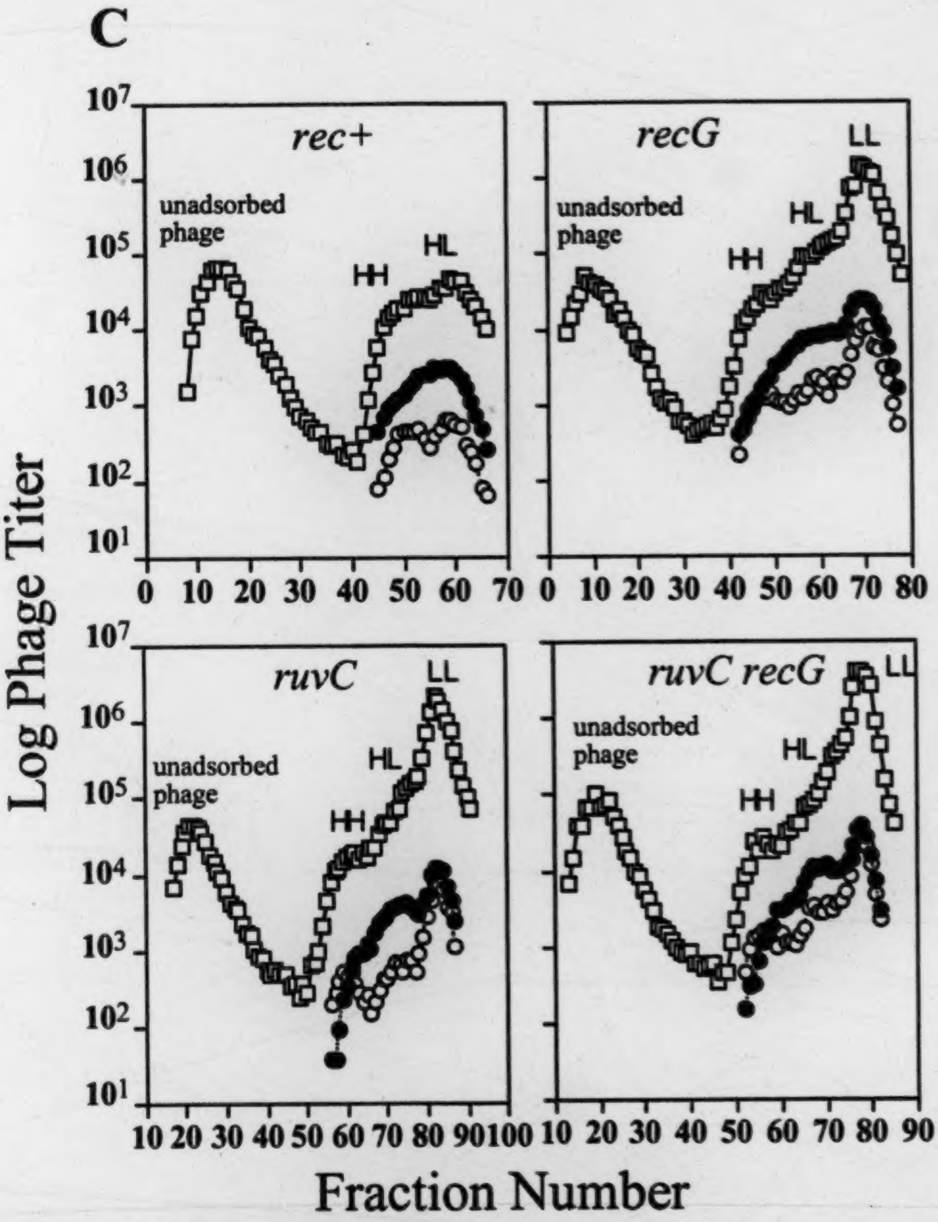


Figure 3-4. An assay to correlate the extent of new DNA synthesis with the interval of recombination in λ crosses performed in cells defective for Ruv or RecG proteins. The crosses were conducted under conditions that allow for some DNA replication to occur (Material and Methods). Density-labeled λ were infected into unlabeled *E. coli* cells. The progeny were examined genetically, to determine the interval of recombination, and physically to detect any new DNA synthesis (Materials and Methods). Replicative central recombinants can be distinguished from break-join central recombinants in a cesium formate density gradient. (*A*) A schematic representation of the phages used in these experiments with their relevant genotypes (Sawitzke and Stahl, 1997). These phages have been used previously (Chapter 2, Sawitzke and Stahl 1997, Materials and Methods), are *red gam*, and carry a deletion for the *nin* region of λ (Δ*nin5*). J^+ S^+ recombinants are selected (Materials and Methods), and from among them central (J^+ *cI* S^+, dark circle) and right end (J^+ cI^+ S^+, open circle) recombinants are counted.



(B) Density gradient of cross progeny from *rec*$^+$, *ruvA*, *ruvB*, and *ruvC* cells. The top curves, represented by open squares, show the titer of total λ, dark circles represent central recombinants (J^+ cI S^+), and open circles represent end recombinants (J^+ cI^+ S^+). The first peak from the left represents unadsorbed phage carrying heavy proteins coats and HH DNA); they are not part of the progeny phage. Three progeny peaks appear: HH, HL, and LL. Even though these crosses were performed under the same conditions for all four strains, we see few or no LL progeny in the *rec*$^+$ graph (discussed in text).



(C) Density gradient of cross progeny from *rec*$^+$, *ruv*C, *rec*G and *ruv*C *rec*G cells. This set of crosses were performed independently of (*B*), but the same symbols are used in these graphs. Note that, again, few or no LL progeny were seen in the *rec*$^+$ graph.

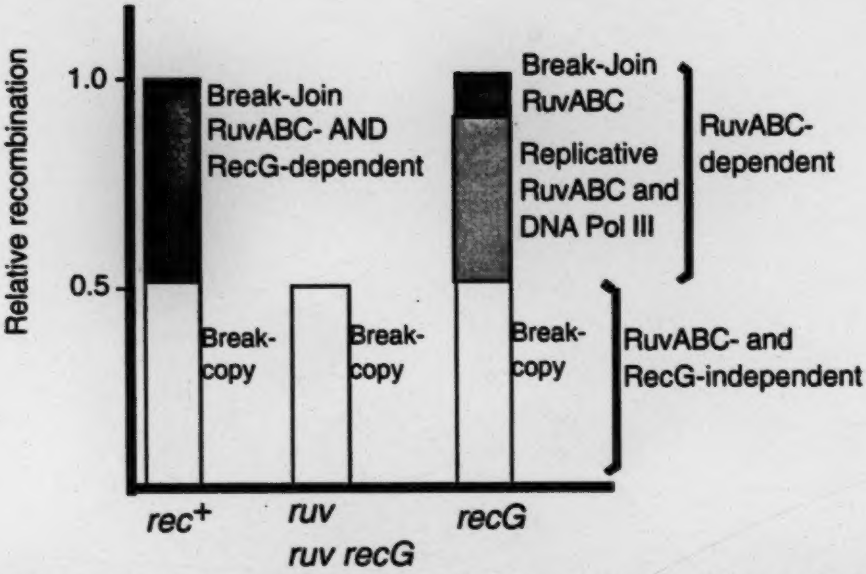


Figure 3-5. A schematic summary of data presented in Chapter 3. This figure depicts the proportion of different λ recombination products recovered from *rec*$^+$, Ruv-defective, RecG-defective, and Ruv-RecG-defective strains and the genetic requirements for their formation. The open rectangles represent the break-copy recombinants which are formed independently of Ruv or RecG proteins, but require DNA synthesis via DNA Pol III. These constitute half of all recombinants recovered from *rec*$^+$ and *recG* cells, and are the only type of recombinants observed in Ruv-defective strains. The black rectangles represent the break-join recombinants whose formation requires RuvABC function, independently of any DNA synthesis. These make-up half of all λ recombinants recovered from *rec*$^+$ cells (in the presence of Ruv and RecG proteins), are not observed in Ruv-defective strains, and form roughly 10-20% of all recombinants seen in *recG* cells. The gray rectangle represents replicated recombination products, whose formation requires RuvABC and DNA Pol III proteins. These are only observed in *recG* cells, constituting roughly 30-40% of all recombinants, and are evidence for a novel replicational recombination mechanism in these cells. These data show that half of recombination requires RuvABC, suggesting the formation of a RuvABC-specific recombination intermediate. RecG is required for the optimal efficiency of RuvABC-mediated break-join pathway; in its absence a RuvABC-dependent replicational mechanism substitutes.

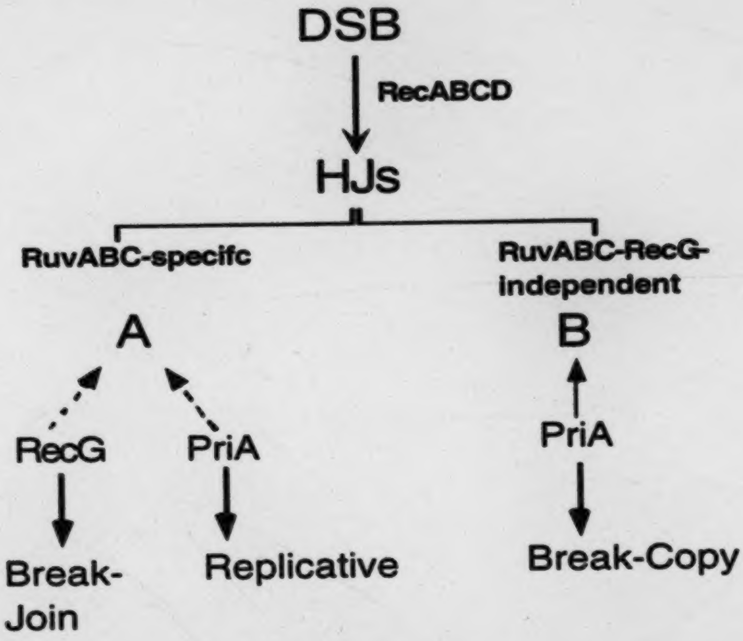


Figure 3-6. A model for the existence of two distinct recombination intermediates in vivo. Two DNA intermediates are formed after the RecABCD-mediated synapse of the homologous molecules ("A" and "B"). These DNA intermediates must be processed in order to form recombinant molecules. I propose that the processing of one of the recombination intermediates (in this case "A") always requires the RuvABC proteins. It may proceed via two mechanisms: in the presence of RecG, "A" is stabilized (by RecG) for processing via RuvABC-dependent break-join pathway. However, because PriA and RecG share binding affinity to similar DNA intermediates, I propose that both can bind to "A", but RecG out competes PriA normally, and thus "A" is processed by the break-join pathway more frequently. If PriA gains access to "A" (e.g. in the absence of RecG), then a replicative pathway is initiated to extend the DNA intermediate, but the formation of a recombination product still requires the RuvABC proteins. The data presented in this report show that in the absence RecG, overall recombination remains constant, but there is a significant decrease in break-join recombination. I propose that a PriA-mediated pathway is compensating for this decrease in break-join recombination. Without the competition from RecG, PriA gains access to this DNA intermediate more frequently, setting up replication forks. I find that the resolution of "A" still requires RuvABC because in *ruv recG* double mutant cells, recombination is half of *rec*$^+$. Also, in *recG* cells, fewer break-join recombinants are observed. I propose that break-join recombination events require RecG to stabilize the DNA intermediate for RuvABC processing. The other recombination intermediate ("B"), is processed via a replicative pathway, requiring DNA Pol III, but neither of the Ruv nor RecG proteins.

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Chapter 4
Conclusions

The general aim of this thesis study was to investigate the role of DNA replication in recombination-dependent double-strand break-repair (DSBR) in the bacterium *Escherichia coli.* DSBR is accomplished via RecBCD-mediated recombination, which is also the main pathway for recombining linear DNA in *E. coli.* Historically, RecBCD-mediated recombination was thought to occur by the breaking and re-joining of homologous molecules, exclusively (e.g. Thaler and Stahl 1988; West 1992; Kowalczykowski et al. 1994; Kowalczykowski 2000). However, recently a plethora of indirect evidence suggested the involvement of DNA replication in the repair of broken DNA chains (e.g. Siegel 1974; Kogoma et al. 1996; Courcelle et al. 1997; Kogoma 1997; Kuzminov and Stahl 1999; Lombardo and Rosenberg 1999). Various replicative models were hypothesized (e.g. Skalka 1974; Smith 1991; Kuzminov 1995; Rosenberg et al. 1996; Kogoma 1997; Kuzminov and Stahl 1999); however, the direct demonstration of newly synthesized DNA within a recombinant molecule was never made.

In Chapter 2, I tested the replicative DSBR hypothesis, by using λ as the DNA substrate, and by physically examining λ recombination products (that have under gone RecBCD-mediated DSBR) for new DNA synthesis. I found genetical and physical evidence that in the absence of HJ processing proteins, Ruv and RecG, RecBCD-mediated repair can occur, but requires DNA replication *via* DNA Pol III. Notably, the physical analysis of recombinant DNA molecules revealed that the break-join pathway is essentially eliminated, and that repair proceeds exclusively via a replicative route in *ruv recG* cells. Other physical evidence (e.g. the accumulation of central recombinant under the HL peak, see Chapter 2 for details) also supports this conclusion. These results show that, with λ as the DNA substrate, replicative repair can occur. Endonucleolytic cleavage of recombination intermediates is not required, but rather resolution is effected via the hypothesized conservative segregation of replicated DNA strands (see

Chapter 2 for a model). However, the question remains: Does this type of repair also occur in *rec*$^+$ cells, or is this a unique pathway activated in cells deficient for HJ processing?

To answer this question, I physically examined recombination products from *rec*$^+$ *E. coli* and found recombinant molecules that also had experienced DNA synthesis. This is the first direct demonstration of a recombination-dependent replicative repair pathway in a non-viral DSBR system. Also, based on the physical and genetical data, I estimate that each pathway (break-join and replicative) is responsible for roughly half of DSBR in wild-type *E. coli*. We proposed a model in which both mechanisms operate in parallel for the efficient repair of DSBs (see Chapter 2).

An unexpected observation was that in the absence of HJ processing proteins a dramatic increase in DNA replication was observed (compared to *rec*$^+$ cells). This result fits well with DSBR models in which DNA recombination intermediates initiate replication forks (e.g. Siegel and Kamel 1974; Skalka 1974; Kogoma 1997); thus the hypothesized accumulation of recombination intermediates in *ruv recG* cells may lead to a large increase in DNA replication.

The conclusions presented above from Chapter 2 directly demonstrate the existence of a DSBR mechanism that operates via DNA replication, requiring DNA Pol III, whose mechanism is different from the previously described break-join pathway. Our results show that this mechanism operates in addition to the break join pathway and requires different proteins.

The discovery of two genetically distinguishable DSBR mechanisms is important, and may have a profound impact on determining the roles of other recombination proteins in *E. coli*. From among the over 20 recombination proteins that have been identified so far, only a few (*e.g.* RecA, RecB, RecC, *etc.*) are thought to have a significant role in the RecBCD-mediated DSBR (reviewed in Kowalczykowski et al. 1994; Kowalczykowski 2000). Others are considered auxiliary and their role *in vivo* remains undetermined. Most confer only a slight decrease in RecBCD-mediated recombination when absent (e. g. Lloyd and Buckman 1995). This fits well with the hypothesis that their role is in one of the two DSBR pathways, such that their absence results in a minor decrease in recombination.

In one sense, the most significant contributions of Chapter 2 were the development of physical and genetical assays that can be used to study each pathway separately. For example, the replicative repair pathway can be studied independently of the break-join pathway by examining repair in *ruv recG* cells; also the break-join pathway can be studied in the absence of the replicative pathway by completely blocking replication in our cells. Furthermore, the ability to physically analyze recombination products for new DNA synthesis is a powerful technology that can be used to test predictions of replicative and break-join models directly, and to build a detailed mechanistic frame-work for DSBR in vivo. This, combined with the biochemical studies of recombination proteins, will have a great impact on our current views of repair in *E. coli* and higher eukaryotes.

In Chapter 3, I employed these techniques to re-examine the role of each HJ processing system (RuvABC and RecG) independently in DSBR in *E. coli*. Previous data, using conjugational and transductional recombination assays, suggested that Ruv and RecG represent two independent (parallel) pathways for processing HJs (Lloyd 1991): the absence of one system results in a slight decrease in recombination, whereas *ruv recG* cells are severely defective for recombination (similar to *recA* cells). I physically examined recombination products recovered from cells defective for a HJ processing protein to determine the role of each protein in DSBR in vivo.

First, I found physical and genetical evidence that break-join recombination requires the RuvABC proteins. In the absence of any Ruv proteins, essentially all break-join recombinants are absent and DNA replication, via DNA Pol III, is required for the remaining recombination. This supports the hypothesis that the RuvABC proteins act as a complex in vivo (supported in vitro by Hiom and West 1995; Eggleston et al. 1997; van Gool et al. 1998; van Gool et al. 1999), required for the break-join DSBR pathway.

Second, I found direct physical evidence that RecG is required for optimal efficiency of the RuvABC-dependent break-join pathway: in its absence, break-join recombination is significantly reduced (3-5 fold). This directly demonstrates that the two previously thought independent pathways for processing recombination intermediates work together to catalyze break-join recombination reactions. I proposed that RecG assists the RuvABC proteins by stabilizing recombination intermediates for RuvABC processing. A molecular explanation for this could be that RecG branch migrates HJs in a direction (left, as shown in Figure 1-2D and 1-2E) that extends the heteroduplex DNA region, stabilizing the D-loop. This activity is hypothesized to be required for the optimal efficiency of RuvABC-dependent break-join recombination in this thesis. In its absence, fewer break-join recombinants are detected as the D-loop may be dismantled by a branch migration activity that removes the heteroduplex region (branch migration to the right, in Figure 1-2E). This means that RecG functions by stabilizing the HJ and presenting this substrate for RuvABC resolution. The biochemistry of RecG accommodates this hypothesis (Whitby et al. 1993; Whitby and Lloyd 1995): RecG has been shown to secure exchanges initiated by 3'-ended invasion via its 3' to 5' helicase activity in vitro.

Third, even though break-join recombination is significantly hindered in *recG* cells, the overall recombination frequency remains unchanged. I proposed that a replicational mechanism compensates for this decrease in break-join recombination, and show that this novel mechanism (operating only in *recG* cells) requires resolution via RuvABC and DNA synthesis via DNA Pol III.

In Chapter 3, I suggest the formation of two distinct recombination intermediates in vivo (see Figure 3-5): one that requires processing via RuvABC (intermediate "A"), and another that is processed via DNA replication, independently of Ruv or RecG proteins (intermediate "B"). I proposed that the RuvABC-specific intermediate is most frequently resolved via the break-join pathway when RecG is present. In the absence of RecG, the intermediate can be extended by PriA-dependent DNA replication, but its maturation still requires the RuvABC proteins. Two assumptions are made in this model: (i) PriA can initiate replication from recombination intermediates (Kogoma et al. 1996; Liu et al. 1999), and (ii) PriA and RecG compete for binding to the RuvABC-specific intermediate shown in vitro (McGlynn et al. 1997) and in vivo (Al-Deib et al. 1996). Both assumptions are supported by results of others. (For a description, see Chapters 1 and 3.) I hypothesize that the mechanism employed for processing of the intermediate depends on the outcome of this competition. If PriA gains access to the RuvABC-specific intermediate ("A"), then replication is used to copy information from the homologous partner, but resolution requires RuvABC; on the other hand if RecG gains access to the intermediate ("A"), RuvABC resolves the intermediate via the break-join pathway.

What might be the nature of these two types of recombination intermediates? Even though these experiments were not designed to address this question, I speculate that the two proposed intermediate, "A" and "B", represent the strand polarity of two different invading ends 3' and 5', respectively. I base my speculation partly on results from a recombination-dependent mutational mechanism operating in *E. coli*, which is RuvABC-specific, RecG-inhibited (reviewed in Rosenberg et al. 1998), and is thought to be mediated via 3'-ended invasion only (Ross et al. unpublished results); and partly on the higher PriA binding affinity to junctions with a 5' tails compared to junctions with 3' tails (Nurse et al. 1999). Both lines of evidence suggest that the RuvABC-specific intermediate ("A") is a 3'-ended single strand DNA invading a duplex. This implies that 5'-ended invasions are targeted by PriA and enjoy replicative DSBR, where as 3'-ended invasions are resolved most frequently via the break-join pathway when RecG is present.

It is also worth noting that D-loops formed via RecA-catalyzed strand invasion often span only a few hundred bases. This may be too small for the assembly of the Pol III enzyme. DNA Pol III is a large multimeric enzyme and is likely to require a larger region of ssDNA for assembly and function than the one available at a D-loop. One way to extend the D-loop for DNA Pol III replication may be to use DNA Pol I as the first step for replication, extending the D-loop and creating a larger region for DNA Pol III assembly and replication. This idea can be tested by using strains that carry a temperature sensitive mutation for DNA Pol I enzymes. If DNA Pol I is required for DNA Pol III-dependent replication of λ chromosome, then no recombinants should be observed at the restrictive temperature, even if the cells are wild type for the DNA Pol III enzyme.

The sophisticated analytical tools available for studying DSBR in *E. coli* are not available in other organisms. In this thesis, I have shown that much can be learned about the molecular details of this process by using these tools. Because many of the *E. coli* DSBR proteins have direct homologues in higher eukaryotes, including man, it is possible that the basic mechanism of repair in *E. coli* is applicable to these organisms.

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